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### 16223

## Determination of Serum Polysaccharides by the Tryptophane Reaction.*

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A number of investigators have described methods for the quantitative estimation of the polysaccharides associated with the serum proteins. Merten[1] estimated these bound sugars by determining the difference between the free sugar and the total reduction after acid hydrolysis of the serum. Lustig and Langer[2] applied the Tillman-Phillippi orcinol method to an alcohol precipitate of serum. Seibert and Atno[3] adopted the carbazole reagent of Dische[4] for the quantitative estimation of polysaccharide in serum, both directly on the proteins obtained by ethanolic precipitation and by difference between the reducing sugar and the total sugar determined with carbazole. Sheppard and Everett[5] described a reaction for the colorimetric determination of carbohydrates by means of tryptophane. The latter determination has the advantage that tryptophane arising from the hydrolysis of protein obviously does not interfere with it. It is the purpose of this paper to describe the application of this reaction to the rapid quantitative assay of non-glucosamine serum polysaccharides.

*Experimental. Reagents:* 1. Sulfuric acid solution, 77% by volume. 770 ml of C.P. concentrated sulfuric acid is added to 230 ml of distilled water.

2. Absolute ethanol.

3. Tryptophane solution, 1%. 1 g of *l*-tryptophane (Eastman Kodak) is dissolved in 99 ml of warm distilled water. The solution is filtered and stored in a glass stoppered bottle in the refrigerator until ready for use.

*Method:* The method for blood serum is as follows: The serum is diluted 1-2 with 0.9% sodium chloride. Two-tenths ml of the diluted serum is pipetted drop by drop into 10 ml of absolute ethanol in glass stoppered centrifuge tubes. The precipitate is centrifuged out and washed once with 10 ml of absolute ethanol. After centrifuging as before, the ethanol is decanted and the precipitate drained by inversion of the tube. One ml of distilled water and 7 ml of 77% sulfuric acid are then added, the tube is allowed to stand at room temperature for 10 minutes, to dissolve the precipitate, and it is then placed in an ice bath for 15 minutes. One ml of cold 1% aqueous tryptophane solution (filtered before using) is added to each tube without shaking. The contents of the tube are mixed and the tube placed immediately in a boiling water

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* Aided by a grant from the Oklahoma Division of the American Cancer Society.

[1] Merten, Richard, *Biochem. Z.*, 1938, **297**, 304.

[2] Lustig, B., and Langer, A., *Biochem. Z.*, 1931, **242**, 320.

[3] Seibert, F. B., and Atno, J., *J. Biol. Chem.*, 1946, **163**, 511.

[4] Dische, Z., *Biochem. Z.*, 1927, **189**, 77.

[5] Sheppard, F., and Everett, M. R., *J. Biol. Chem.*, 1937, **119**, 1.

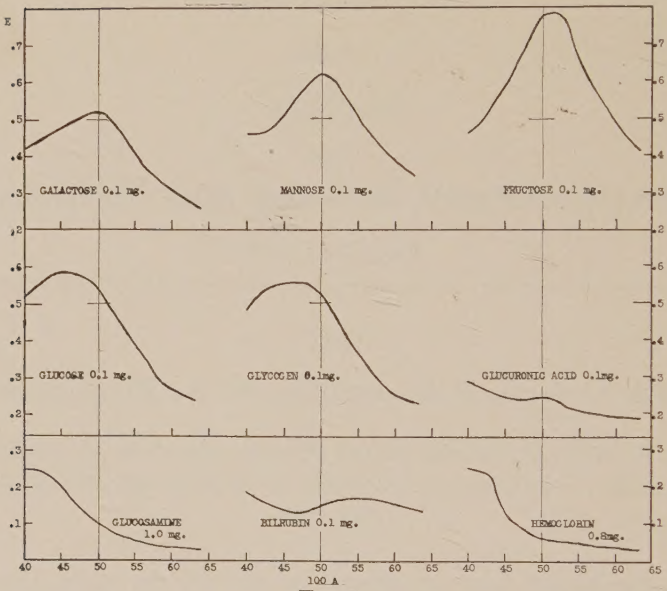

FIG. 1.

Absorption curves of single components after reaction with tryptophane.

bath for a period of 20 minutes. The tube is shaken once after it has been heated 10 minutes. After removing from the boiling water the tubes are cooled for 5 minutes in an ice bath and then allowed to stand 25 minutes at room temperature. The concentration is determined in a Coleman No. 11 spectrophotometer by reading against a blank (without the serum precipitate) which is run through the above procedure.

*Standard:* Equal amounts of *d*-galactose and *d*-mannose were used as a standard since present evidence indicates that the polysaccharide of serum is composed of equimolecular amounts of glucosamine, mannose, and galactose.[6] As shown by absorption curves (Fig. 2) glucosamine has little influence on readings made at 500 mμ, increasing the absorption only 1%.

*Results. Absorption Curves.* Absorption curves for mannose, galactose, glucose, glycogen, fructose, glucosamine, glucuronic acid, bilirubin, and hemoglobin are shown in Fig. 1. The maximum absorption for galactose and for mannose is at 500 mμ, for fructose at 520 mμ, for glucose at 460 mμ. As expected, glucose and glycogen produce similar curves.

Absorption curves of mixed sugars are shown in Fig. 2. The optical densities of the individual constituents are additive within experimental error. A typical serum absorption curve, also absorption curves for ascitic fluid, and hydrocele fluid are included in Fig. 2. All curves have been calculated to the same height at 500 mμ. It is noteworthy that the serum, ascitic fluid, and hydrocele fluid curves differ from the galactose-mannose-glucosamine curve by their greater absorption in the 400-480 mμ range. However, absorption curves of partially purified serum polysaccharide (polysaccharide: total nitrogen ratio = 0.97 : 1) and a similar polysaccharide from hydrocele fluid (polysaccharide: total nitrogen ratio = 0.33 : 1) resemble closely the standard galactose-mannose-glucosamine curve. The enhanced absorption between 400 and 480 mμ is therefore due to the serum protein.

When the above described procedure is followed, except that 1 ml of distilled water is added to the serum precipitate in place of the tryptophane solution and the result is read against a blank containing 80 γ of tryptophane (the amount present in 0.0667 ml of

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[6] Meyer, K., *Adv. in Protein Chemistry,* 1945, **11**, 249.

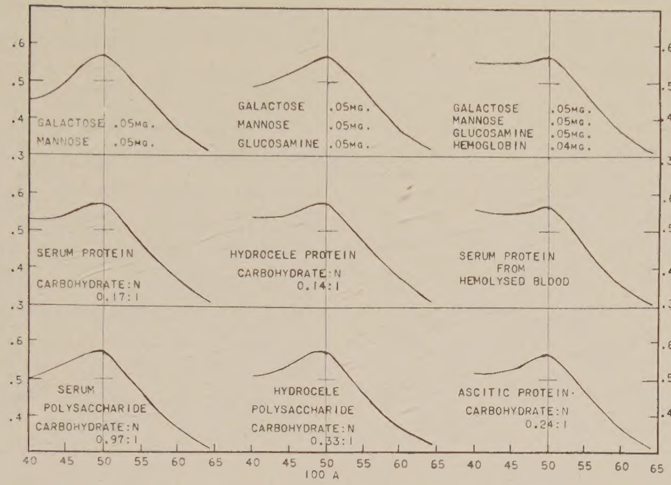


FIG. 2.

Absorption curves of sugar mixtures and of body fluids after reaction with tryptophane.

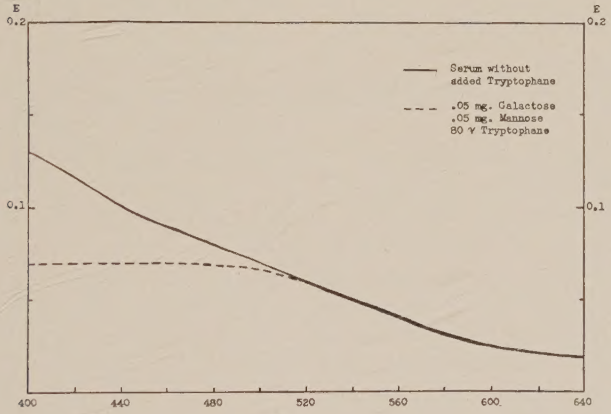


FIG. 3.

Absorption curve of serum protein without added tryptophane read against 60% sulfuric acid.

serum containing 7% protein) the upper absorption curve in Fig. 3 is obtained. The lower curve was obtained by allowing a standard solution of galactose, mannose, and glucosamine to react with 80 γ tryptophane under the above conditions. The difference between the two curves should then represent the result of chromophoric reactions of the system other than the tryptophane-sugar reaction. When this derived curve is used to correct the serum absorption curve, they approach the absorption curves of the galactose-mannose-glucosamine mixtures within experimental error. It is noteworthy that no appreciable correction is necessary for the absorption at 500 m$\mu$. The nature of the substance or substances in serum protein which increase absorption in the region between 400 and 480 m$\mu$ was not discovered. Addition to standard sugar solutions of tyrosine, cystine, and histidine at levels found in serum protein failed to influence the shape of the galactose-mannose-glucosamine curve.

Hemolysis of the blood sample, if extensive, results in a positive error; the effect of hemoglobin on the absorption curve (Fig. 2) is most striking in the 400-480 m$\mu$ range and small at 500 m$\mu$. Therefore, the polysac-

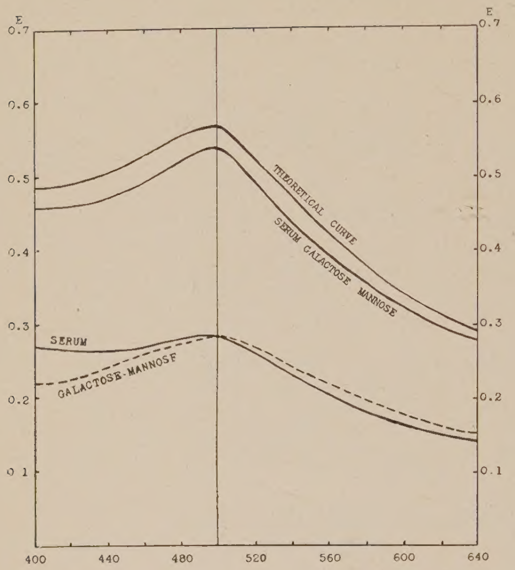
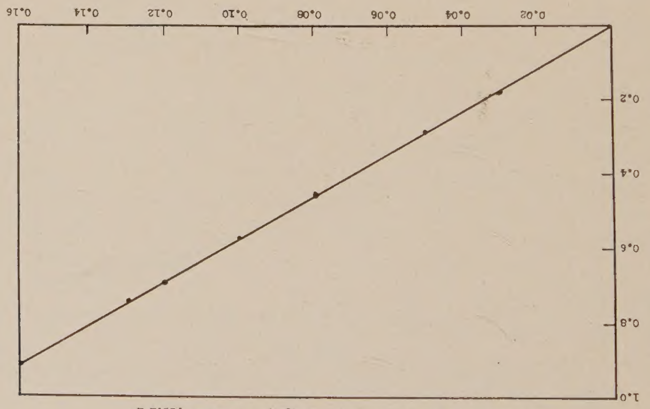


FIG. 4.
Effect of added sugar upon serum-tryptophane absorption curve.



Milligrams of Galactose and Mannose.
FIG. 5.
Concentration curve of the sugar-tryptophane system at 500 mμ.

charide of slightly hemolysed samples may be determined with reasonable accuracy.

*Recovery Curves.* Mixtures of standard galactose and mannose with serum gave the absorption curve shown in Fig. 4. The height of this curve varies between 94% and 97% of the calculated value obtained by adding the absorption curve for the sugars to that of the serum.

*Efficiency of the Precipitation Process.* Since it was necessary to learn if any free sugar is carried down in the ethanolic precipitation, glucose was determined on aqueous solutions of the precipitates of several serums using the Folin-Wu procedure with a Somogyi (zinc hydroxide) precipitation. No glucose was found in any of these precipitates.

To determine the completeness of the polysaccharide precipitation the total carbohydrate and the polysaccharide were determined by the tryptophane method on the same samples of serum. Free sugar was also determined on these serums by the Folin-Wu procedure using the Somogyi precipitation. When the difference in absorption between the total carbohydrate and the polysaccharides was calculated as glucose, the results are as follows:

| | Serum 1 | Serum 2 |
|---|---|---|
| Polysaccharide by tryptophane method | 132 | 118 |
| Glucose by Folin-Wu method | 97 | 102 |
| Total carbohydrate (calculated) | 229 | 220 |
| Total carbohydrate by tryptophane method | 228 | 224 |

Since the total carbohydrate figures determined directly or by calculation agree quite closely and since the precipitate contains no glucose, the serum polysaccharides are apparently completely precipitated by the procedure described.

*Calculations.* In the concentration range, 0.02 mg to 0.16 mg of an equimolecular mixture of galactose and mannose, the Lambert-Beers law applies,as indicated in Fig. 5. Calculation of unknown concentrations may be made by either reading from the concentration curve or by the following formula:

$$\frac{Ex}{Es} \times .1 \times \frac{100}{.0667} = \text{mg\% polysaccharide as galactose and mannose}$$

where Ex is the extinction (log 1/transmission) of the unknown, and Es is the extinction of 0.1 mg of an equimolecular mixture of galactose and mannose.

*Effect of Aging on Absorption Curves.* When the solutions were read at various times after development of color, absorption curves similar to those shown in Fig. 6 were obtained. During the first 30 minutes, absorption increased in the 400-480 mμ range. No significant change in the curve occurred between 30 and 60 minutes. Consequently, for quantitative results the samples were read between 30 and 60 minutes after removal from the hot water bath.

*Effect of Heating Time on Color Development.* When the heating time in the boiling water bath was varied between 10 and 30 minutes, the absorption curves shown in Fig. 7 resulted. There is a progressive increase in the absorption at both ends of the curve as the heating time is increased. Little variation occurred at 500 mμ except in the 25-minute curve for the galactose-mannose mixture.

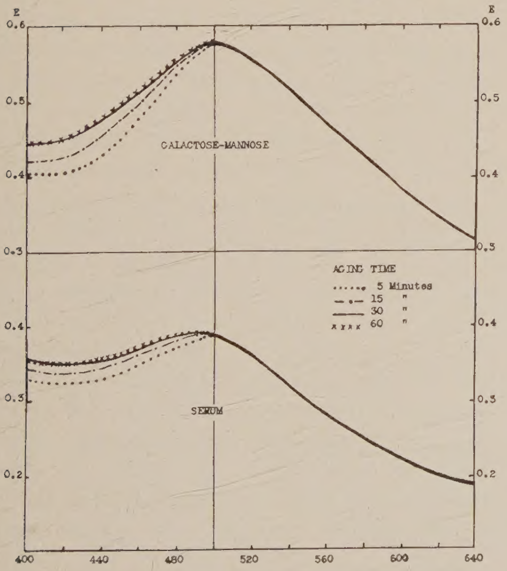


Fig. 6.

The effect of aging after removal from the boiling water bath upon the absorption of the sugar-tryptophane system.

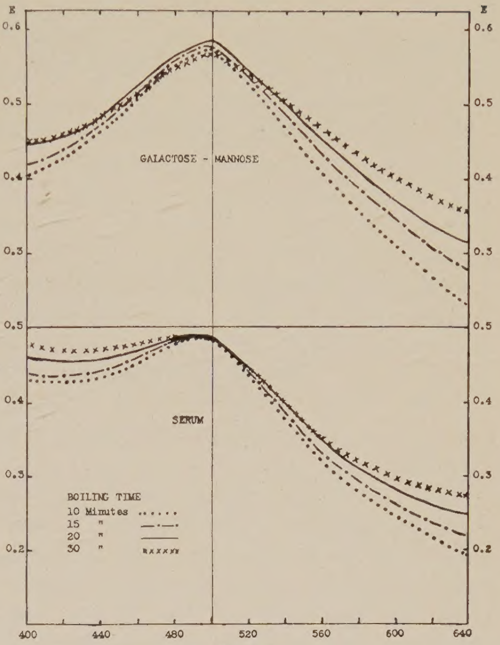


Fig. 7.

The effect of heating time on the absorption of the sugar-tryptophane system.

*Quantitative Results on Human Sera.* The concentrations of polysaccharide, exclusive of glucosamine, in the sera of 59 normal persons representing both sexes and all ages varied between 80 mg % (fetal blood) and 139 mg % (blood from a senile man). These values are in general agreement with those of Siebert.[3] Detailed results from studies of normal and pathological subjects will be given in another paper. The method described has considerable accuracy, the average difference between duplicates being 2.4% in a large number of blood serum samples.

*Summary.* A colorimetric method based on the reaction of tryptophane with carbohydrates is described for the determination of serum non-glucosamine polysaccharide. The absorption maximum for the carbohydrate-tryptophane compound is at 500 mμ for galactose and for mannose, 460 mμ for glucose, and 520 mμ for fructose. Glucuronic acid, glucosamine, and hemoglobin have maxima at wave lengths below 400 mμ. Glucuronic acid also exhibits a weak band at 480-500 mμ.

The absorption curves for alcohol precipitates from serum, ascitic fluid, and hydrocele fluid differ from postulated galactose-mannose-glucosamine curves by showing greater absorption in the 400-480 mμ range. This difference diminishes when the polysaccharide is partly freed from protein.

Hemolysis, if marked, introduces a positive error in the method. The color produced by the carbohydrate tryptophane reaction obeys the Lambert-Beers law within the limits investigated.

### 16224

## Oxygen Uptake of Human Placental Tissue as Affected by Selected Substrates and Drugs.*

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Very few human tissues other than the placenta are readily available for *in vitro* studies. Despite this fact, little is known about the influence of metabolites or of pharmacologic agents upon this tissue. Any knowledge that may be gained about the behavior of this complex organ, furthermore, may assist in understanding some unsolved problems of human reproduction. We have selected for study the substrates commonly used for the *in vitro* study of tissue metabolism, and those drugs most frequently employed in obstetrics.

Early reports upon the oxygen uptake of human placental tissue[1-4] gave very low $Q_{O_2}$ values, perhaps attributable to their methods. Loesser[5] studied the oxygen consumption, anaerobic and aerobic glycolysis and in placentas two months of age found values for $Q_{O_2}$ of 5.0, $Q_L^{N_2}$ of 7.0 and $Q_L^{O_2}$ of 5.1. At term, these values were 1.4, 3.5, and 0.57, respectively. Wang and Hellman[6] demonstrated changes in placental $Q_{O_2}$ with relation to age in a manner corresponding well with the histologic changes in the villi. The $Q_{O_2}$ was found to decrease from 5.3 at the second month to 1.7 at term, and the addition of glucose to the medium did not alter this value. No reports have been found regarding the influence of any other agents upon this tissue.

*Methods.* Placentas were placed in an ice-packed container immediately following delivery. The tissue was prepared for respiration studies in a cold box at 5°C.[7] In choosing the experimental material necrotic, highly calcified, or traumatized areas were avoided. Thick sagittal slices were immersed in Ringer's solution and the villi teased free with dissecting needles. Samples of villi weighing from 200 to 250 mg were blotted on filter paper, weighed, and transferred to Warburg flasks. Conventional manometric methods were used throughout these studies. The medium consisted of 2.0 ml of Krebs-Ringer-phosphate solution (pH 7.3) modified to conform more closely to the composition of interstitial fluid.[8] The substrate was 0.2% glucose unless otherwise specified, and the gas phase was oxygen. The oxygen consumption is expressed as microliters of oxygen per milligram dry weight tissue per hour ($Q_{O_2}$). In testing the effect of drugs upon oxygen uptake, a control period of 80 minutes preceded the experimental

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* Aided by grants from the John and Mary Markle Foundation, New York City, and the National Institute of Health, Bethesda, Md.

[1] Rech, W., *Z. f. Biol.*, 1924, **80**, 231.

[2] Kustner, H., and Siedentopf, H., *Arch. Gynak.*, 1929, **138**, 131.

[3] Adler, K., *Arch. Gynak.*, 1930, **140**, 338.

[4] Matsushita, D. T., *Mitt. a. d. med. Akad. zu Kioto*, 1935, **13**, 872.

[5] Loesser, A., *Arch. Gynak.*, 1932, **148**, 118, and **148**, 123.

[6] Wang, H. W., and Hellman, L. M., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 31.

[7] Fuhrman, F. A., and Field, J., II, *J. Biol. Chem.*, 1944, **153**, 515.

[8] Elliott, H. W., Warrens, A. E., and James, H. P., *J. Pharm.*, 1947, **91**, 98.

period of 80 minutes, with readings obtained at 20-minute intervals. The "normal" $Q_{O_2}$ for each placenta is the average $Q_{O_2}$ of all flasks for the first 80 minutes.

*Results.* The substrates studied were dextrose, sodium pyruvate, sodium succinate, and hydroquinone. The mean $Q_{O_2}$ of 29 placentas delivered within 2 weeks of the estimated date of confinement was found to be 1.92 ± a standard deviation of 0.373. The presence of dextrose (0.011 M) made no significant difference in the rate of oxygen uptake. The $Q_{O_2}$ could be maintained for 5 hours with a decline of only 13%. Similar results were obtained with sodium pyruvate (0.045 M). The addition of sodium succinate (0.017 M), however, gives an initial $Q_{O_2}$ of 3.6, with a decline of 34% in 2 hours and 66% in 4 hours. Increasing the succinate concentration to 0.2 M raised the initial $Q_{O_2}$ to 7.3, with a decline of 23% in 2 hours. With hydroquinone in concentrations of 0.045 M, 0.091 M, 0.182 M, and 0.364 M, the initial $Q_{O_2}$ values were 16.4, 20.8, and 25.4 respectively. In 2 hours, all 4 values had fallen 78%. For any substrate studied, the figures represent the means of at least 6 observations on 3 placentas. While both succinate and hydroquinone are oxidizable substrates, the maximal $Q_{O_2}$ obtainable with the former is limited by the concentration of enzymes in the succinoxidase system, whereas the maximal rate obtainable with hydroquinone (after correction for auto-oxidation) is limited only by the cytochrome-cytochrome oxidase system.

The drugs selected for study were merperidine (Demerol), methadon (10820, amidone, Dolophine), morphine, Amytal, and scopolamine. Observations were also made on diethylstilbestrol and dinitrophenol. Their effects upon the oxygen uptake of placental tissue are summarized in tabular form.

It will be noted that merperidine, Amytal, and scopolamine have only a depressant action, whereas methadon has a stimulating action in the lower concentrations. Morphine has no influence upon oxygen uptake. Dinitrophenol has the usual marked stimulating effect, while diethylstilbestrol, in aqueous suspension, has the same depressant effect noted on homogenates of liver and pituitary[9] or upon cellular suspensions of rat brain.[10]

*Discussion.* These studies indicate that the behavior of placental tissue in the Warburg flask is similar in many respects to other mammalian tissues. Like muscle or kidney, placental tissues contain stored substrates, since neither glucose nor pyruvate increase the $Q_{O_2}$ above the non-substrate value. In common with most tissues, placental $Q_{O_2}$ in the presence of succinate or hydroquinone is dependent, within limits, upon substrate concentration and is much higher than that obtained with such carbohydrate substrates as glucose or pyruvate.

The actions of the drugs tested agree qualitatively with effects reported for brain and other tissues. If the mechanism of drug action is likewise similar, it may be assumed that merperidine and methadon inhibit respiration by their action on certain dehydrogenases involved in the oxidation of carbohydrates, whereas Amytal exerts its inhibitory action at a point between the flavoproteins and cytochromes. Comparisons of placenta with other tissues may be made by reference to the remarks in Table I.

With the exception of Amytal, it will be noted that the concentrations of drugs employed are far in excess of the probable therapeutic concentration. It would not be anticipated, therefore, that the employment of such agents in the usual amounts would affect the total oxygen consumption of the placenta *in vivo*. It should be pointed out, furthermore, that the influence of a drug upon placental respiration is not related to the influence which that same drug may have upon the respiratory centers of the fetus. Finally, it must be recognized that our present state of knowledge does not permit us to surmise what effects, if any, a stimulation, whether by succinate or dinitrophenol administration, or a moderate drug depression of oxygen uptake would have upon the multiple functions of placental transfer and secretion.

*Summary.* The rate of oxygen consumption of fresh human chorionic villi was meas-

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[9] McShan, W. H., and Meyer, R. K., *Arch. Biochem.*, 1946, **9**, 165.

[10] Gordan, G. S., and Elliott, H. W., *Endocrin.*, 1947, **41**, 517.

TABLE I.
Effect of Drugs upon Oxygen Uptake of Human Placental Tissue *in vitro.*

| Drug | *In vivo* conc.* | *In vitro* conc. | Maximal effect on $Qo_2$ (in % change from control value) | Comparison with other tissues |
|---|---|---|---|---|
| Morphine | 2 x $10^{-6}$ M | 2.5 x $10^{-4}$ M | 0 | As found for brain slices.[8] |
| | | 5.0 x $10^{-3}$ M | 0 | |
| Merperidine | 1.8 x $10^{-5}$ M | 2.5 x $10^{-4}$ M | —10 | Approximately 10 times as sensitive to merperidine as brain slices.[8] |
| | | 5 x $10^{-4}$ M | —27 | |
| | | 1 x $10^{-3}$ M | —43 | |
| | | 7.5 x $10^{-3}$ M | —93 | |
| Methadon | 2 x $10^{-6}$ M | 2.5 x $10^{-4}$ M | +22 | Post stimulatory inhibition at low concentrations not as marked as with brain slices.[8] Similar to diaphragm.[11] |
| | | 5 x $10^{-4}$ M | +38 | |
| | | 1 x $10^{-3}$ M | +12 | |
| | | 2 x $10^{-3}$ M | —93 | |
| Scopolamine | 8 x $10^{-8}$ M | 2.5 x $10^{-4}$ M | —11 | Initial inhibition followed by partial recovery after 80 minutes.† |
| | | 5 x $10^{-3}$ M | —21 | |
| | | 2 x $10^{-2}$ M | —56 | |
| Amytal | 5 x $10^{-5}$ M | 1 x $10^{-4}$ M | 0 | Similar to effects of Amytal on brain slices.[12] |
| | | 2 x $10^{-4}$ M | — 9 | |
| | | 4.5 x $10^{-4}$ M | —31 | |
| | | 9 x $10^{-4}$ M | —63 | |
| | | 1.8 x $10^{-3}$ M | —97 | |
| Dinitrophenol | — | 4 x $10^{-6}$ M | +56 | Usual effect of dinitrophenol. |
| Stilbestrol | — | aq. suspension 4 mg/vessel | —88 | As found for brain homogenates.[10] |

* Estimated extracellular concentration after therapeutic dose.
† Data for tissues other than human placenta not available.

ured by conventional manometric methods. The addition of dextrose or pyruvate did not influence the $Qo_2$ whereas succinate and hydroquinone increased the rate in proportion to their concentrations.

The $Qo_2$ of human placental tissue is depressed by merperidine, Amytal, scopolamine and diethylstilbestrol and stimulated by dinitrophenol. Morphine had no effect, while methadon stimulated respiration in low concentrations and depressed it at higher levels. None of the drugs exerted an effect in therapeutic concentrations.

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[11] Bachelor, A. L., and Elliott, H. W., *Fed. Proc.*, 1948, **7**, 203.

[12] Fuhrman, F. A., and Field, J., II, *J. Pharmacol.*, 1943, **77**, 392.

### 16225

# Quantitative Distribution of an Esterase Among Cytoplasmic Components of Mouse Liver Cells.*

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It was previously demonstrated by Dounce[1] that nuclei of rat liver cells, isolated by differential centrifugation of cell suspensions treated with citric acid, contained a concentration of esterase which was about 50% of that found in the whole tissue; methyl butyrate was used as the substrate. If the ratio of nuclear to cell volume is taken to be 6:100 for hepatic cells, as approximated by Marshak,[2] then the separated nuclei contain only around 3% of the esterase activity of the cell. It is therefore apparent that a study of the cytoplasmic components of the liver cell is essential to an understanding of the intracellular localization of the esterase. The unusually high concentration of esterase in the liver lends interest to an investigation of this nature since the special accumulation of the enzyme in this tissue can hardly be considered a merely fortuitous circumstance. Just which of the many esterifications and de-esterifications known to take place in the liver are catalyzed by this enzyme still remains to be elucidated. The distinct differences in the nature and properties of liver esterase and lipase have been known for some time.[3]

The quantitation of the activities observed in the various separated fractions as related to the activity of the whole cell has been emphasized in the present investigation since, as Danielli[4] has pointed out, the centrifugal segregation of cellular components may result in an alteration of the enzymatic activities as they exist *in situ* through separation of naturally occurring inhibitors, activators, etc.

*Experimental.* Livers from 2 or 3 female $ZAF_1$ strain mice were used in each experiment. The portal vein was clamped prior to the removal of the organ, ether anesthesia being used throughout. In the subsequent procedures up to the point of the determination of enzyme activity, care was taken to maintain the tissue as close to 0°C as possible.

The whole livers were put through a tissue press and weighed. The material was transferred to a mortar and ground thoroughly for 4 minutes with the slow addition of 4 volumes of alkalized physiological saline solution (2 cc of 0.1N NaOH per liter of 0.85% NaCl). The diluted tissue sample was further ground in a Potter-Elvehjem homogenizer[5] for about 20 seconds. This ground tissue preparation was assumed to be a 5 times dilution of the original tissue sample.

The differential centrifugation procedure employed was essentially that of Claude[6] with some modifications and additions and is summarized in Fig. 1. The washings indicated in the figure were carried out with volumes of physiological saline solution equal to that of the supernate in each case. The values of (g) were calculated from the center of the centrifuge tube in every instance. The time for the whole separation procedure from the initial removal of the liver was approximately 3 hours. Esterase activity was determined by a manometric method similar to that of Rona and Lasnitzki[7] in a Warburg apparatus. 0.5 cc of the enzyme solution was placed in the sidearm of the reaction flask and 1.5 cc of

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* Aided by a grant from the Medical Research Fund of the Graduate School of the University of Minnesota.

[1] Dounce, A. L., *J. Biol. Chem.*, 1943, **147**, 685.

[2] Marshak, A., *J. Gen. Physiol.*, 1941, **25**, 575.

[3] Sobotka, H., and Glick, D., *J. Biol. Chem.*, 1934, **105**, 199.

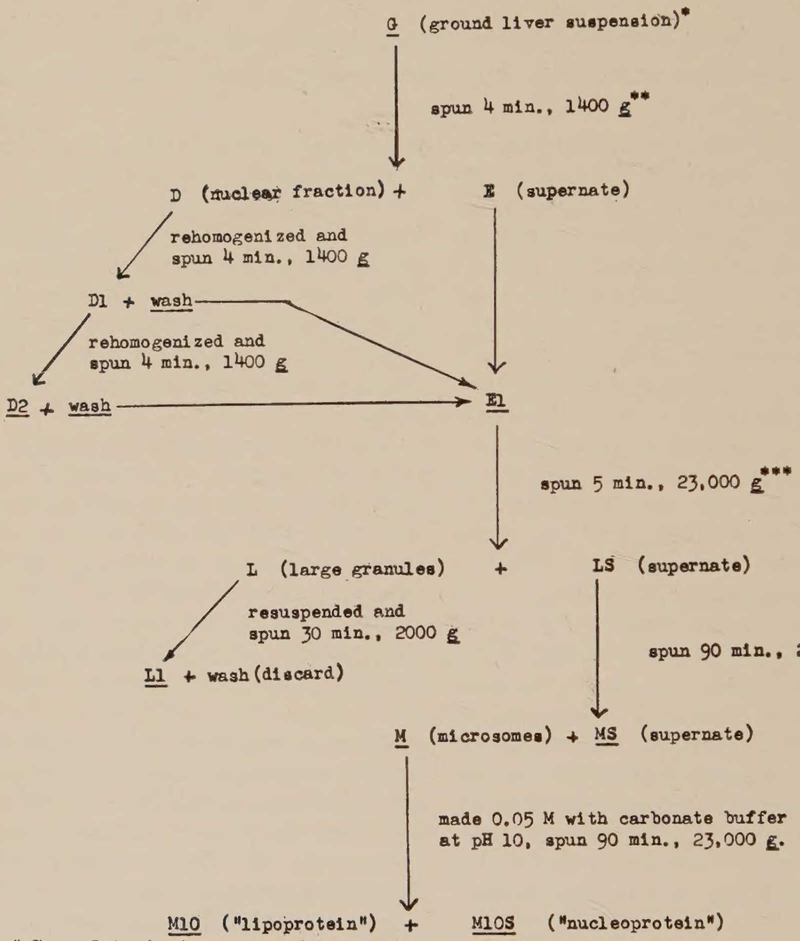
[4] Danielli, J. F., *Nature*, 1946, **157**, 755.

[5] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

[6] Claude, A., *J. Exp. Med.*, 1946, **84**, 51.

[7] Rona, P., and Lasnitzki, A., *Biochem. Z.*, 1924, **152**, 504.

FIGURE I. FRACTIONATION OF LIVER CELL SUSPENSION BY DIFFERENTIAL CENTRIFUGATION


G (ground liver suspension)*
spun 4 min., 1400 g**
D (nuclear fraction) +
E (supernate)
rehomogenized and spun 4 min., 1400 g
D1 + wash
rehomogenized and spun 4 min., 1400 g
D2 + wash
E1
spun 5 min., 23,000 g***
L (large granules) +
LS (supernate)
resuspended and spun 30 min., 2000 g
L1 + wash (discard)
spun 90 min., 23,000 g
M (microsomes) + MS (supernate)
made 0.05 M with carbonate buffer at pH 10, spun 90 min., 23,000 g.
M10 ("lipoprotein") +
M10S ("nucleoprotein")

* Ground 4 min. in mortar with 4 volumes of alkalized physiological saline solution, then ground for 20 seconds in Potter-Elvehjem homogenizer.

** Servall angle centrifuge operated in refrigerator.

*** Multi-speed attachment to International centrifuge operated in cold room at —15°C.

bicarbonate-Ringer solution was introduced into the main chamber of the vessel. 0.022 cc of methyl butyrate was pipetted into the main chamber; the final concentration of 1% of the substrate in the complete reaction mixture thus obtained gives the optimum rate of hydrolysis without excess-substrate inhibition. The control vessel contained 0.5 cc of bicarbonate-Ringer solution in the sidearm in place of the enzyme solution. A 5% $CO_2$, 95% $N_2$ gas mixture was passed through the system before the contents were mixed. Nitrogen determinations were conducted by the Pregl micro-Kjeldahl method.

*Results.* The results are shown in Tables I and II. The unit of enzyme activity was chosen as the amount of enzyme which liberates 1 $\mu$l of $CO_2$ per hour at pH 7.4, 37.5°C and at the optimum substrate concentration. In the initial separation of the D and E fractions the cytoplasm was shown to contain most of the esterase of the cell. The N content is about 2.5 times, the enzyme activity per g of wet liver tissue is almost 5 times,

TABLE I.
Esterase Distribution Among Fractions of Mouse Liver Cell.

| Exp. | 1 | | | 2 | | | 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Fraction | N yield, % of G | Enz. conc., units/μg N | Enz. yield, % of G | N yield, % of G | Enz. conc., units/μg N | Enz. yield, % of G | N yield, % of G | Enz. conc., units/μg N | Enz. yield, % of G |
| G | 100 | 16.3 | 100 | 100 | 13.8 | 100 | 100 | 10.4 | 100 |
| D2 | 29 | 7.6 | 14 | 27 | 9.4 | 19 | 26 | 6.8 | 17 |
| E1 | 64 | 20.2 | 80 | 67 | 13.5 | 65 | 70 | 12.6 | 85 |
| L1 | 11 | 23.2 | 15 | 10 | 23.1 | 17 | 12 | 17.5 | 20 |
| M | | | | 10 | 52.9 | 39 | 12 | 47.0 | 55 |
| MS | | | | 42 | 3.8 | 12 | 46 | 3.3 | 15 |
| M10 | | | | | | | 6 | 55.0 | 34 |
| M10S | | | | | | | 6 | 19.0 | 10 |

Note: N content of E and G were found to be 2.2% and 3.8%, respectively, based on wet weight of liver tissue.

TABLE II.
Summation of Nitrogen and Enzyme contents of Various Fractions.

| | Nitrogen | | | | | | Enzyme | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | D2 + E1 | | L1 + M + MS | | M10 + M10S | | D2 + E1 | | L1 + M + MS | | M10 + M10S | |
| Exp. | Found | Theory | Found | Theory | Found | Theory | Found | Theory | Found | Theory | Found | Theory |
| 1 | 93 | 100 | | | | | 92 | 100 | | | | |
| 2 | 94 | 100 | 62 | 67 | | | 84 | 100 | 68 | 65 | | |
| 3 | 96 | 100 | 70 | 70 | 12 | 12 | 102 | 100 | 90 | 85 | 44 | 55 |

and the enzyme activity per μg N is around 2 times as great in the E fraction as in the D2. From the study of the N and enzyme distribution among the particulate fractions of the cytoplasm, Table I, it is clear that, although the greatest part of the N was confined to the supernate, MS, from the microsome separation, the largest portion of the enzyme was contained in the microsome fraction, M.

It has been shown that the separation of the M fraction into "lipoprotein" (M10) and "nucleoprotein" (M10S) fractions can be effected by treatment with a carbonate buffer at pH 10.[8] The separation of the M into M10 and M10S fractions resulted in an equal distribution of N, however, the enzyme was concentrated in the M10 portion. These results were confirmed in duplicate experiments.

Examination of the data reveals that the separation of the various particulate fractions does not result in a noticeable loss of enzyme activity. This is shown by totaling the N values and enzyme activities found in the various sub-fractions and comparing the sums with the theoretically expected values. For example, the sum of the N values found in the L1, M and MS fractions should approximate the observed E value. These comparisons are summarized in Table II. The fact that the summation of enzyme activities approximated the theoretical values indicates that naturally occurring activators or inhibitors of the esterase in the mouse liver cell, if present, were not separated from the enzyme by the fractionation procedures.

It is possible that the presence of considerable activity in the L1 fraction and some activity in the D2 fraction may be derived from contamination of these fractions with microsomes. Electron microscopic examinations of the L1 fraction in other comparable experiments appear to lend weight to this possibility. The presence of some intact cells is another possible source of the activity in the D2 fraction. The esterase activity found in our nuclear fraction (D2) therefore cannot be compared with Dounce's[1] values for isolated hepatic nuclei.

The isolation of pure nuclei with 1% citric acid, essentially the method of Marshak,[2] was carried out in one experiment, but the enzyme activity found was negligible. The esterase concentration in serum was also determined and was found to be less than 1% of that in the liver tissue. Hence no appreciable activity could be ascribed to contamination with serum.

*Summary.* The esterase in the cytoplasm of mouse liver cells was found to be localized predominantly in the microsome fraction which contained an average of 47% of the enzyme in the whole tissue and 63% of that in the entire cytoplasm. Other cell fractions, the nuclear material, the large cytoplasmic granules, and the supernate from the microsome separation contained an average of 17%, 17%, and 14%, respectively, of the enzyme in the whole tissue. Enzyme activity per μg of N was greatest in the microsomes which contained a concentration that was 4.2 times that of the whole tissue. In the other particulate fractions, the nuclear material, the large cytoplasmic granules, and the supernate from the microsome separation, the enzyme concentration was found to be 0.6, 1.6, and 0.3 times, respectively, that of the whole tissue. The "lipoprotein" fraction of the microsomes displayed about 3 times the enzyme concentration found in the "nucleoprotein" fraction. Summation of the enzyme content of the substituent fractions indicated no appreciable loss during the separation procedures.

We are indebted to Dr. J. J. Bittner for the mice used in these experiments.

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[8] Barnum, C. P., and Huseby, R. A., unpublished data.

16226

# Lipids of the Fasting Mouse. IV. Liver Total Lipid Content.

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The inter-relation of liver and muscle in the complex mechanisms of fat utilization have often been considered; the fasting mouse has proved to be a valuable object of study in this connection.[1,2] Despite the rapid mobilization of available carcass fat and its prompt metabolism in the first days of fasting, the liver weight decreases in a regular fashion so that approximately half its initial weight is lost during a four day fast. The liver total lipid in this period exhibits a striking fatty accumulation followed by a reduction to below normal values.

In earlier studies, considerable variation had been observed in mortality, in liver weight loss and in accumulation of total lipid in the liver. Thus, a mortality of 66% was reached in 4 fasting days when the room temperature was occasionally permitted to fall even from 25° to 20° C, whereas only 22% mortality had occurred by the same day when the room temperature was held at 25° C. In the case of liver weight, the losses had been sufficiently irregular so that no simple tendency could be described. As to the liver total lipid, in one study the amount present had approximately doubled after 1 day of fasting but thereafter decreased to less than normal amounts; however, with more careful room temperature control and longer survival, the liver total lipid content had remained elevated on both the second and third fasting days. Finally, it was desired to extend the period of observation from 4 to 5 fasting days. With these several problems in mind, the following experiment was undertaken.

*Experimental.* The fasting procedure in general was as described before.[1] Three months old, male, albino mice previously maintained on a diet of oats and Purina dog chow were fasted in individual cages. Water was supplied *ad lib.* and the room temperature was kept at 25°C. The mice were lightly anesthetized with ether and sacrificed by bleeding from the axillary artery.[3] The livers were removed quickly and analyzed by standard procedures for total lipid.[4]

*Mortality.* No mice died during the first 2 fasting days; thereafter the mortality rapidly increased to about 50% on the fifth fasting day (see Table I). The temperature was maintained at 25°C in the animal room; at this temperature, the results reflected a mortality rate similar to that previously seen. A lower room temperature is known to be less favorable to survival.

*Body Weight Loss.* The average initial body weight varied from 20 to 22 g; the extreme variation among individual mice was 19 to 24 g. The body weight when recorded at the time of sacrifice was calculated as the sum of the carcass weight, the liver weight, and the weight of the blood sample. An almost linear decrease in body weight was observed with fasting. The average percentage body weight losses for the 2 preceding series[1,2] and for the current experiments were almost identical. The average mouse lost about a third of its original weight

TABLE I.
Percentage Mortality: The Relation to Room Temperature Control.

| Room temperature °C | Days of fasting 1 | 2 | 3 | 4 | 5 | |
|---|---|---|---|---|---|---|
| 20-25 | 0 | 13 | 39 | 66 | — | (1) |
| 25 | 0 | 0 | 0 | 22 | — | (2) |
| 25 | 0 | 0 | 10 | 55 | 50 | (current) |

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[1] Hodge, H. C., MacLachlan, P. L., Bloor, W. R., Stoneburg, C. S., Oleson, M. C., and Whitehead, R., *J. Biol. Chem.*, 1941, **139**, 897.

[2] MacLachlan, P. L., Hodge, H. C., Bloor, W. R., Welch, E. A., Truax, F. L., and Taylor, J. D., *J. Biol. Chem.*, 1942, **143**, 473.

[3] Kuhn, L. R., *Science*, 1941, **93**, 504.

[4] Bloor, W. R., *J. Biol. Chem.*, 1928, **77**, 53.

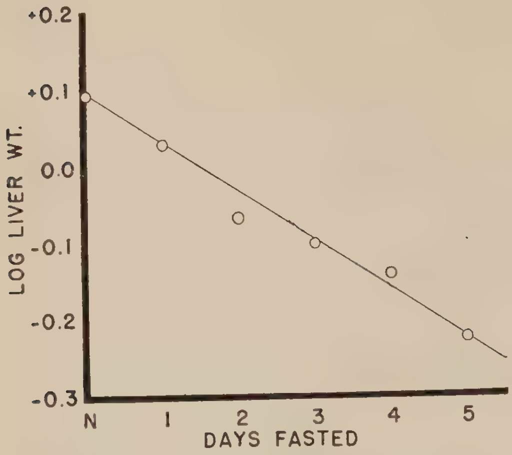

Fig. 1.

Logarithm (base 10) of moist liver weight in g plotted against the days fasted. The linear tendency is evident.

by the fifth day. The calculated equation for the average percentage body weight loss based on nearly 300 fasting mice is as follows: % weight loss = 1.10 + 7.48 × days fasted.

*Liver Weight Loss.* A logarithmic decrease in liver weight was found (Fig. 1); the liver weight dropped from about 5.5% of the body weight in the normal mouse to 2.9% of the initial body weight in the mouse fasted 5 days. This corresponds to a loss of about 52% of the initial liver weight. The fact that the liver loses weight more rapidly than the carcass has been repeatedly observed (Table II).

*Liver Total Lipid.* The milligrams of total lipid for whole liver increased 2- or 3-fold during the first 2 fasting days (Table III); thereafter, it dropped to an amount significantly less than normally present. Expressed as percentage of *moist* liver weight the total lipid values are as follows: normal—7%, fasted one day—12%, two days—17%, three days—7%, four and five days—6%. One mouse after one day's fast had 17% fat in its liver; 2 mice on the second fasting day had 28 and 29%, respectively, of liver fat. The increase in liver total lipid has been shown to be due to an increase in the neutral fat fraction and to occur despite a decrease in the phospholipid content. It can be reasonably assumed that the fat which appeared in the fasting liver had its origin in a mobilization of depot fat.

*Summary.* Young adult male albino mice

TABLE II.
Data on Body and Liver Weights of the Normal and Fasting Mice.

| | | Body weight | | | | Liver weight | |
|---|---|---|---|---|---|---|---|
| | | Initial | | Carcass | | | |
| | No. mice | Mean (g) | S.D. | Mean (g) | S.D. | Mean (g) | S.D. |
| Normal | 15 | 20 | 1.3 | 18.7 | 1.5 | 1.099 | 0.16 |
| Fasted 1 day | 18 | 21 | 1.2 | — | — | 0.920 | 0.08 |
| ” 2 ” | 15 | 21 | 0.8 | — | — | 0.836 | 0.21 |
| ” 3 ” | 15 | 20 | 0.8 | 14.6 | 1.3 | 0.865 | 0.20 |
| ” 4 ” | 22 | 22 | 0.9 | 15.6 | 1.3 | 0.696 | 0.10 |
| ” 5 ” | 11 | 20 | 1.0 | 12.2 | 1.0 | 0.578 | 0.08 |

TABLE III.
Combined Summary Data on Liver Moist Weight and Total Lipid of Normal and Fasting Mice.

| | Liver moist weight* | | | Liver total lipid† | | |
|---|---|---|---|---|---|---|
| Condition | No. | Avg, g | S.D. | No. | Avg, mg | S.D. |
| Normal | 55 | 1.24 | 0.26 | 34 | 70 | 17 |
| Fasted 1 day | 58 | 1.06 | 0.15 | 37 | 108 | 30 |
| ” 2 ” | 55 | 0.85 | 0.18 | 35 | 123 | 56 |
| ” 3 ” | 55 | 0.79 | 0.17 | 34 | 51 | 24 |
| ” 4 ” | 62 | 0.71 | 0.13 | 39 | 32 | 16 |
| ” 5 ” | 11 | 0.58 | 0.08 | 10 | 38 | 5 |

* Data from references 1 and 2 plus current data.
† Data from reference 2 plus current data.

lose about one-third of the initial body weight during a 5 day fast. The liver weight decreases logarithmically. In the first 2 days of fasting a marked increase was observed in total liver lipid of young, male mice. Thereafter a decrease to less than normal values followed on the third to fifth fasting days.

## 16227

## Studies on Q Fever: Complement-Fixing Antibodies in Meat Packers at Fort Worth, Texas.*

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Previous studies[1,2] have suggested that risk of infection with Q fever is greater among stockyard and packinghouse workers than among the general population. The recovery of *Rickettsia burneti* from ticks in Liberty County, Texas,[3] and the more recent demonstration of the same organism as the causative agent of a natural outbreak of Q fever among stock handlers and slaughterhouse workers at Amarillo, Texas,[4] stimulated interest in the possibility of unrecognized human infections in this area. Accordingly, a serologic survey was undertaken to determine the frequency of complement-fixing antibodies to *R. burneti* in a group of packing plant employees at Fort Worth, Texas.

Serum specimens were obtained from the laboratory of the Fort Worth Health Department. The blood samples were collected during May and June, 1947, for the performance of routine serological tests for syphilis, from employees of meat packing plants. The sera used in this study were obtained only from workers who handled meat or meat products in raw or prepared states. Of the 1,433 specimens examined, approximately ¾ were secured from employees of one large packing house, the remainder being obtained from workers at a number of smaller establishments. When the routine tests for syphilis were completed, the sera were transported to this laboratory and stored in the deep freeze cabinet until tested for antibodies against *R. burneti.*

The Q fever antigen† was prepared from yolk sacs of embryonated eggs infected with a strain of *R. burneti* (American Nine Mile) isolated in Montana in 1935.[5] The antigen consisted of a washed rickettsial suspension prepared by a combination of ether extractions to remove the fats and repeated centrifugation cycles to free the rickettsial bodies from extraneous proteins and fats. All tests

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* These studies are being aided by grants from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y., and the Rose Lampert Graff Foundation, Los Angeles, Calif.

[1] a. Derrick, E. H., *Med. J. Australia,* 1937, **2**, 281; b. Freeman, M., Derrick, E. H., Brown, H. E., Smith, D. J. W., and Johnson, D. W., *Australian J. Exp. Biol. and Med. Sc.,* 1940, **18**, 193; c. Derrick, E. H., *J. Hyg.,* 1944, **43**, 357.

[2] Shepard, C. C., *Am. J. Hyg.,* 1947, **46**, 185.

[3] Parker, R. R., and Kohls, G. M., *Pub. Health Rep.,* 1943, **58**, 1510.

[4] a. Topping, N. H., Shepard, C. C., and Irons, J. V., *J. A. M. A.,* 1947, **133**, 813; b. Irons, J. V., and Hooper, J. M., *J. A. M. A.,* 1947, **133**, 815; c. Irons, J. V., Murphy, J. N., and Wolfe, D. M., *J. A. M. A.,* 1947, **133**, 819; d. Cox, H. R., Tesar, W. C., and Irons, J. V., *J. A. M. A.,* 1947, **133**, 820.

† The authors are indebted to Dr. Herald R. Cox, Virus and Rickettsial Section, Lederle Laboratories Division, American Cyanamid Company, for generously supplying the yolk sac antigen (lot No. Q 9M-5-16) used in these studies.

[5] Davis, G. E., and Cox, H. R., *Pub. Health Rep.,* 1938, **53**, 2259.

were performed with a single lot of antigen. Complement-fixation tests were carried out according to the method described by Bengtson.[6] The antigen was diluted 1:32 (2 units). Complement was titrated in the presence of antigen; 2 units of complement were used in the tests.

Serial 2-fold dilutions of inactivated sera were made, using a single pipette for each serum specimen. The hemolytic system consisted of equal volumes of 2% sheep erythrocytes and amboceptor made up to 2 units. In performing the tests, 0.2 cc of diluted antigen, serum, and complement were added to each tube, mixed thoroughly, and stored at ice-box temperature for 18 hours. Sensitized sheep erythrocytes (0.4 cc) were then added, and the tubes held at 37°C in a water bath for one hour or until control tubes showed complete hemolysis. Appropriate serum and antigen controls, as well as known positive and negative human and guinea pig sera, were included in each series of tests. The end point was read as the highest serum dilution showing at least 3+ fixation.

All sera were examined in a "screen" test using 2 dilutions of serum: 1:8 and 1:16. Sera having titers of 1:16 in the "screen" test were retested in 2-fold serial dilutions from 1:8 to 1:512 or greater.

Complement-fixing antibody titers against the *R. burneti* antigen are recorded in the accompanying table. Of the 1,433 specimens examined, 81 were anti-complementary and 1,238 showed less than 3+ fixation in a serum dilution of 1:8. One hundred and fourteen of the sera (8.0%) showed titers of 8 or greater, while 80 (5.6%) had a titer of 16 or more and 32 (2.3%), 32 or more. Seventeen serum specimens (1.2%) showed titers of 64 or more; among these, 10 had a complement-fixing antibody titer of 64, 2 a titer of 128, 3 a titer of 256, and 2 a titer of 512.

TABLE I.
Complement-Fixation Titers Against *R. burneti* in Sera from Meat Packers at Fort Worth, Texas.

| Titer* | No. of serum specimens | % | Total % positive |
|---|---|---|---|
| AC | 81 | 5.7 | |
| <8 | 1238 | 85.7 | |
| 8 | 34 | 2.4 | 8.0 |
| 16 | 48 | 3.3 | |
| 32 | 15 | 1.1 | |
| 64 or > | 17 | 1.2 | |
| Total | 1433 | 99.5 | |

* Highest serum dilution yielding 3+ or greater fixation.

AC Serum anticomplementary.

The individuals with antibodies for Q fever worked in various departments of the plants and there was no clear evidence that the positive reactors were engaged in any particular job. Some suggestive evidence was found that the higher titers occurred in individuals dealing with cattle rather than in those engaged in work with hogs and sheep. Epidemiological significance cannot be attached to these findings since the total number of individuals employed or tested in each department at the time of the study is unknown.

Among all the specimens examined, serological tests for syphilis[‡] (Kahn, Kolmer) were positive in 39 instances (2.7%) and doubtful in 19 (1.3%). Among the 114 sera showing complement-fixing antibody titers of 8 or more against *R. burneti*, 4 (3.5%) had positive serological tests for syphilis and 2 (1.8%) had doubtful tests. The complement-fixation titers for *R. burneti* among these 6 individuals with positive or doubtful serological tests for syphilis were 8 in 2 instances, 16 in 3, and 512 in 1. The findings suggest only a coincidental relationship between serum reactivity with antigens for syphilis and Q fever.

*Discussion.* Considerable evidence exists to indicate that the complement-fixation test with yolk-sac antigens of *R. burneti* is reliable and specific for the serologic diagnosis of Q fever. In proved infections in which *R. burneti* was recovered from the blood stream, a rise in titer of complement-fixing antibodies has been demonstrated during convalescence.[4,7] Serological cross-reactions with other rickettsial diseases do not occur;[4,6,7] but it should be noted that there is little available information with regard to possible cross-reactions with other non-rickettsial diseases, with the exception of primary atypical pneumonia.[7,8] However, agglutination tests with *R. burneti* have been negative in a variety of other diseases.[9]

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[6] Bengtson, I. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 665.

[‡] These tests were performed by Mrs. Edith Mazurek in the laboratory of the Fort Worth Health Department.

It is not yet certain whether non-specific complement-fixation may occur with egg yolk sac antigens of *R. burneti*. In this laboratory a number of sera which reacted with the egg yolk sac antigen of *R. burneti* were also tested with an antigen prepared from uninoculated normal egg yolk sacs, but in no instance was complement fixation accomplished. Non-specific complement-fixation with syphilitic sera has been reported with a variety of other egg yolk sac antigens.[10,11] Further studies with regard to these problems are in progress in this laboratory.

The significance that can be attached to the results of a complement-fixation test for Q fever on a single serum sample is uncertain, particularly when the serum titer is low (8 or 16) and when a clinical history of illness is unavailable. Early in convalescence considerably higher titers are to be expected[2,4,7] but at longer intervals after infection lower titers might be anticipated. Elsewhere, however, it has been demonstrated that elevated serum antibody titers may persist for 1½ years after natural infection with Q fever.[12] Illness due to Q fever may vary in severity from a trivial attack of "grippe"[13] to fatal pneumonia,[4] and subclinical infection may result in elevated antibody titers.[2,4] It is apparent, therefore, that a history of illness may have little value in the interpretation of antibody titers obtained by serological survey. In the present study, the plant physicians were unaware of any unusual prevalence of undiagnosed febrile illness.

The results of the studies herein reported are believed to indicate the existence of unrecognized exposure to *R. burneti*. Similar data have not been reported in this country from other geographic areas or in other occupational groups. At present no conclusion can be drawn as to whether this frequency of occurrence of antibodies for Q fever is unusual. The occupation of these individuals may be immaterial and it is possible that a similar prevalence of antibodies for Q fever may be found in other occupational groups or in the general population in this area or elsewhere. Studies designed to elucidate some of these problems are in progress. Recent studies have indicated the occurrence of Q fever among persons exposed to dairy cattle in the Los Angeles area. *R. burneti* has also been isolated from the milk of such cattle.[14]

*Summary*. Complement-fixation tests employing an egg-yolk sac antigen of *R. burneti* (American Nine Mile strain) were performed on sera from 1,433 packinghouse workers at Fort Worth, Texas. Evidences of previous infection with *R. burneti* were obtained. Antibody titers of 8 or more were found in 8.0%, 16 or more in 5.6%, 32 or more in 2.2%, and 64 or more in 1.2%.

The authors are indebted to Dr. Harold M. Williams and Mrs. Edith Mazurek of the Fort Worth Health Department for their kind cooperation. The complement fixation tests were performed by Elizabeth L. Watson.

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[8] Dyer, R. E., *Am. J. Hyg.*, 1944, **39**, 308.

[9] Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1944, **44**, 110.

[10] Wertman, K., *J. Lab. and Clin. Med.*, 1945, **30**, 112.

[11] Van der Scheer, J., Bohnel, E., and Cox, H. R., *J. Immunol.*, 1947, **56**, 365.

[12] Sulkin, S. E., and Strauss, E., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 142.

[13] Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1946, **44**, 123.

[14] Huebner, R. J., *et al.*, *Public Health Rep.*, 1948, **63**, 214.

## 16228

# Studies On Q Fever: Persistence of Complement-Fixing Antibodies After Naturally Acquired Infection.*

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Most of the information available with regard to the persistence of complement-fixing, agglutinating, or neutralizing antibodies in Q fever has come from the study of infections acquired accidentally by laboratory workers. Bengtson[1] demonstrated the persistence of complement-fixing antibodies in the sera of 2 laboratory workers 10 months after infection with Q fever. Huebner[2] reported that 14 individuals infected with Q fever in 1940 at the laboratory of the National Institute of Health escaped infection in the 1945-1946 outbreak. However, some of these individuals presumably were exposed to the agent in the laboratory at various times between the 1940 and 1946 outbreaks, and in addition, some of them may have been immunized with a vaccine which, it is believed, had some protective effect against *R. burneti.* In the Q fever outbreak in the Respiratory Diseases Commission laboratory,[3] rickettsial agglutinin titers tended to decline after 2 months but in some cases were detectable 3 to 4 months after infection.

Little information is available concerning persistence of antibodies following Q fever acquired under natural conditions. Australian workers[4] demonstrated rickettsial agglutinins in the sera of 2 individuals 17 months after naturally acquired Q fever; complement-fixation tests were not performed.

An outbreak of Q fever acquired under natural conditions at Amarillo, Texas, in March, 1946,[6] presented an opportunity to obtain data on the persistence of complement-fixing antibodies against *R. burneti.* It was felt that such data would be of value in the interpretation of the results of serological surveys for Q fever.[5]

The technique of the complement-fixation test, employing as antigen† yolk sacs of embryonated eggs infected with *R. burneti* (American Nine Mile), is described elsewhere.[5] The end point of the test was read as the highest serum dilution showing at least 3+ fixation.

Post-convalescent blood specimens were obtained from a group of 17 persons who were ill with Q fever in March, 1946. The diagnoses were established on the basis of clinical illness compatible with Q fever, isolation of *R. burneti* from the blood of several patients, and a rise in titer during convalescence of complement-fixing or neutralizing antibodies for *R. burneti.*[6] The blood samples obtained 5 to 7 weeks after onset of illness were tested in the laboratories of Dr. Herald R. Cox of Lederle Laboratories Divi-

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* These studies are being aided by grants from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y., and the Rose Lampert Graff Foundation, Los Angeles, California.

[1] Bengtson, I. A., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 665.

[2] Huebner, R. J., *Am. J. Pub. Health,* 1947, **37**, 431.

[3] Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1946, **44**, 123.

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† The authors are indebted to Dr. Herald R. Cox, Virus and Rickettsial Section, Lederle Laboratories Division, American Cyanamid Company, for generously supplying the yolk sac antigen (lot No. Q 9M-5-16) used in these studies.

TABLE I.
Persistence of Complement-Fixing Antibodies Against *R. burneti* Antigen in Subjects Ill with Naturally-Acquired Q Fever.

| | Titer at indicated time after illness | | |
|---|---|---|---|
| Subject | 5-7 wks | 6 mo.* | 17 mo. |
| 1 | 1024 | >32 | 64 |
| 2 | 256 | >32 | 64 |
| 3 | 128 | 16 | 32 |
| 4 | 64 | — | 16 |
| 5 | + | — | 128 |
| 6 | + | — | 16 |
| 7 | + | — | 1024 |
| 8 | 64 | 32 | — |
| 9 | 256 | 32 | — |
| 10 | 256 | >32 | — |
| 11 | 256 | >32 | — |
| 12 | 128 | >32 | — |
| 13 | 512 | < 8† | — |
| 14 | 128 | >32 | — |
| 15 | + | >32 | — |
| 16 | + | 32 | — |
| 17 | + | 8 | — |

Titer recorded as highest serum dilution yielding 3+ or greater fixation.

* Not tested in dilution greater than 1:32.

+ Positive C.-F. test; titer unknown.

† 8 was lowest serum dilution measured.

— Not tested.

sion, American Cyanamid Company, and Dr. J. V. Irons, Texas State Health Department. Elevated titers of complement-fixing antibodies were present for 17 months after infection, as shown in the accompanying table. In all but 3 subjects tested 6 months after illness, titers of 32 or greater were obtained; in only one subject did the titer fall below 8. Likewise, 17 months after infection, titers of 32 or more were obtained in the serum of 5 of the 7 individuals tested.

All of the complement-fixation tests were performed with antigens of the same strain of *R. burneti* prepared from the infected yolk sacs of embryonated eggs. However, different lots of antigen were used for the tests on the early (5-7 weeks) convalescent specimens and the late or post-convalescent specimens (6 and 17 months). It is recognized that exact comparison cannot be made of titers obtained in different laboratories or even in the same laboratory at different times. Nevertheless, experience has indicated that the general trend of the titers has comparative significance.

*Discussion.* The demonstration that high titers of complement-fixing antibodies may persist for 1½ years after naturally acquired Q fever is important in both clinical and epidemiological studies of this disease. The clinical diagnosis of Q fever is difficult, particularly in sporadic cases. Diagnosis depends largely on the isolation of the rickettsia or on the results of serological studies. Because antibodies may persist for long periods the results of serological studies made during convalescence from a presumed attack of Q fever may be misinterpreted. As with other serological tests, the demonstration of a rise in antibody titer in convalescent phase as compared with acute phase blood specimens is of greater diagnostic value than a high titer present in a single specimen obtained during convalescence.

Little is known of the natural mode of infection with Q fever in this country. It is, therefore, conceivable that the persistence of elevated titers of antibodies in the subjects tested may be the result of continued contact with the rickettsial agent, subsequent to the initial illness. The unusual high titer in case No. 7, referred to in the table, suggests some such mechanism.

To determine whether or not Q fever may be endemic in the Amarillo area, 175 blood specimens from presumably normal individuals were tested for complement-fixing antibodies. These blood samples were collected for routine serological tests for syphilis and were obtained from the Amarillo City Health Department laboratory. Approximately 8% of these specimens showed complement-fixing antibodies against *R. burneti*. Only 2 individuals, however, had high titers (1:64 or more) and it was subsequently determined that both of these individuals had had Q fever during the epidemic. Clinical histories of previous illness were not available from other subjects who exhibited positive complement-fixation in low titers, and it is not possible, therefore, to assess the significance of these reactions.

*Summary.* Complement-fixation tests using an antigen of *R. burneti* (American Nine Mile) were performed on sera from a group of persons known to have had Q fever in 1946 in Amarillo, Texas. High titers of complement-fixing antibodies were demonstrated 17 months after illness.

The authors are indebted to Dr. John M. Hooper and Mr. Jack Wyatt of the Amarillo City Health Department for their kind cooperation. The complement-fixation tests were performed by Elizabeth Lee Watson.

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## 16229

# Effect of Digitoxin On Creatinine and Histamine Output of the Isolated Heart.*

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(Introduced by E. M. K. Geiling.)
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In the course of some experiments on the isolated hearts of rabbits, cats and rats which were perfused with digitoxin to measure the amounts of fixation of the drug, some unexpected observations were made. In using the modified Baljet reaction[1,2,3] to determine the digitoxin colorimetrically, it was found that the intensity of the color in the perfusate was increased over that produced by the digitoxin in the perfusion fluid. This suggested the possibility that creatinine, which also gives the Baljet reaction, might be present in the perfusate. This was found to be the case. It was also observed that the perfusate contained a substance which caused a drop in the blood pressure of an anesthetized cat and a contraction of the isolated guinea pig intestine suspended in atropinized Tyrode solution. This led to the supposition that the perfusate contained histamine. Various investigators have found that the contracting heart muscle produces histamine;[4,5,6] however, this observation could not be confirmed by other workers.[7] Since the creatinine and histamine output of the heart muscle might be related to its physiological activity, it seemed worth while to perform experiments in which the release of these substances could be correlated with the action of cardiac glycosides.

*Experimental.* The effect of digitoxin in a final concentration of 1:40,000 was studied on isolated rat, cat, and rabbit heart preparations. The animals were sacrificed by a blow on the head, and the heart was immediately removed and washed in oxygenated Ringer-Locke solution. The hearts were perfused through the coronary arteries by means of a cannula inserted into the aorta. The temperature of the perfusion fluid was kept at 36-39°C. Samples of perfusate were collected at intervals before and after the introduction of digitoxin into the system. Digitoxin was dissolved in 95% alcohol. Five ml of this solution contained 25 mg which diluted to 1000 ml gives a final concentration of 1:40,000. Control measurements showed that this amount of alcohol did not affect the motility of the heart and did not influence the creatinine and histamine output. The volume of fluid perfused per unit of tissue was not affected by digitoxin. In the case

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† Hoffman La Roche Fellow.

‡ Guggenheim Fellow.

[1] Bell, J. C., and Krantz, F. K., *J. Pharm. and Exp. Therap.*, 1945, **83**, 213.

[2] Krantz, F. K., and Bell, J. C., *Fed. Proc.*, 1947, **6**, 346.

[3] Elmquist, A., and Liljestrand, A., *Acta Physiol. Scandinav.*, 1946, **12**, 53.

[4] Eisa, E. A., *J. Roy. Egyptian M. A.*, 1947, **30**, 189.

[5] Marcu, I., *C. R. Soc. de Biol.*, 1939, **130**, 573.

[6] Anrep, G. V., Barsoum, G. S., and Talaat, M., *J. Physiol.*, 1936, **86**, 431.

[7] Code, C. E., Evans, C. L., and Gregory, R. A., *J. Physiol.*, 1938, **92**, 344.

TABLE I.
Creatinine Excretion of Isolated Hearts.

| | | Samples (Creatinine gamma per 10 ml per g heart) | | | | | |
|---|---|---|---|---|---|---|---|
| Animal | | 1 | 2 | 3 | 4* | 5* | 6* |
| Rabbit | Exper. | 0.48 | 1.14 | 1.03 | 1.30 | 1.08 | 0.89 |
| | Control | 0.68 | 0.63 | 0.89 | 0.83 | 0.61 | 0.85 |
| Cat | Exper. | 0.76 | 0.52 | 0.43 | 0.73 | 0.82 | 0.74 |
| | Control | 1.23 | 0.92 | 0.76 | 0.47 | 0.84 | 0.62 |
| Rat | Exper. | 3.72 | 1.38 | 1.22 | 3.55 | 1.88 | 1.84 |
| | Control | 3.14 | 2.35 | 5.01 | 2.03 | 2.05 | 5.21 |

* After digitoxin (1:40,000) perfusion started.

of the rabbit and cat hearts, the time which elapsed between the introduction of digitoxin and complete arrest of the heart were noted. The more resistant rat hearts were allowed to beat for an hour with digitoxin perfusion before the experiment was discontinued. The hearts were weighed at the end of each experiment. Ten ml samples of perfusate were collected for creatinine and histamine analyses. The samples were kept at ice-box temperature and analyses were carried out afterwards.

The substance in the perfusate which gave the Baljet reaction was identified as creatinine by use of the NC bacterial enzyme suspension of Dubos and Miller.[8,9] The Jaffe alkaline picrate reaction was applied to samples of the perfusate before and after incubation for 90 minutes at 37°C with micro-organisms capable of destroying creatine and creatinine under aerobic conditions. The true creatinine was calculated from the difference in the color reaction before and after incubation. Two 4 ml aliquots of each perfusate sample were taken; one was subjected directly to colorimetric reaction with 2 ml of alkaline picrate, whereas the other was incubated for 90 minutes with 1 ml of cell suspension and 0.25 ml of M phosphate buffer, pH 7.0. Since the amount of protein present was less than could be precipitated by tungstate, the preparation of a tungstate filtrate was omitted. Care was taken to use a cell suspension of potency sufficient to metabolize all the creatinine present. After incubation, separation was carried out by centrifugation. To 4 ml of the supernatant liquid, 2 ml of alkaline picrate was added. The alkaline picrate solution was prepared from 1 volume of 10% NaOH in 5 volumes of freshly saturated picric acid. After the solution had been allowed to stand for 15 minutes, it was added to standard and unknown solutions in a ratio of 1 alkaline picrate to 2 of the other solution. The color was allowed to develop for 10 minutes and was then measured using a Coleman Universal Spectrophotometer, Model 11, with the PC-4 filter, and at a wave length of 525 m$\mu$. A solution of 1 volume of alkaline picrate and 2 volumes of distilled water was used as the blank. The creatinine content of the perfusates was calculated from the readings on standard creatinine solutions and expressed in gamma per 10 ml of perfusate per gram of heart.

The histamine determinations were carried out as follows. Essentially the histamine extraction method of Barsoum and Gaddum[10] was applied. Ten ml samples of perfusion fluid were acidified with 1 ml concentrated HCl. The acidified perfusate was boiled for 2 hours, then evaporated to dryness, and extracted with 5 ml 95% ethyl alcohol saturated with NaCl. The alcohol was evaporated, and the residue taken up in 2 ml of water. This solution was tested on the atro-

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[8] Miller, B. F., and Dubos, R., *J. Biol. Chem.*, 1937, **121**, 429, 447, and 457.

[9] Miller, B. F., Allinson, M. J. C., and Baker, Z., *J. Biol. Chem.*, 1939, **130**, 383.

[10] Barsoum, G. S., and Gaddum, G. H., *J. Physiol.*, 1935, **85**, 1.

TABLE II.
Statistical Analysis of Creatinine Results.

| No. of animals | No. of samples Before | No. of samples After | Mean values Before | Mean values After | T values* |
|---|---|---|---|---|---|
| 10 Rabbits | 21 | 22 | 0.97 | 0.90 | 1.4 |
| 10 Cats | 21 | 20 | 0.69 | 0.73 | 1.5 |
| 7 Rats | 14 | 16 | 4.54 | 3.84 | 1.9 |

* Significance of differences between the means; less than 3.0 is not considered statistically significant.

$$T = \frac{M_1 - M_2}{\sqrt{E_1^2 - E_2^2}} \quad (E = \text{mean error, } M = \text{mean}).^{11}$$

TABLE III.
Histamine Excretion of Isolated Hearts.

| Animal | | Samples (Histamine, gamma per 10 ml per g heart) 1 | 2 | 3 | 4* | 5* | 6* |
|---|---|---|---|---|---|---|---|
| Rabbit | Exper. | .034 | .036 | .033 | .015 | .008 | .000 |
| | Control | .030 | .026 | .030 | .028 | .028 | .030 |
| Cat | Exper. | .019 | .020 | .017 | .009 | .007 | .003 |
| | Control | .014 | .018 | .010 | .015 | .013 | .018 |
| Rat | Exper. | .11 | .11 | .12 | .11 | .11 | .11 |
| | Control | .08 | .07 | .12 | .11 | .10 | .10 |

* See Table I.

TABLE IV.
Statistical Analysis of Histamine Results.

| No. of animals | No. of samples Before | No. of samples After | Mean values Before | Mean values After | T values* |
|---|---|---|---|---|---|
| 11 Rabbits | 26 | 25 | .033 | .0095 | 6.71 |
| 10 Cats | 16 | 18 | .0135 | .0053 | 7.52 |
| 10 Rats | 22 | 23 | .13 | .13 | 0.00 |

* See Table II.

pinized guinea pig intestine in the usual way. The sensitivity of the intestine was frequently tested with known amounts of histamine. This extraction method proved to be quite useful for estimating histamine in perfusates containing digitoxin. The method eliminates digitoxin and potassium ions, which, if present in the original perfusate, cause contraction of the guinea pig ileum.

*Results.* The results are summarized in tables wherein typical experimental data and the statistical analyses of a large number of experiments are presented. The creatinine output in each of the 3 species investigated was fairly constant and not affected by digitoxin (Tables I and II). The highest creatinine output was observed in rat hearts. The creatinine outputs of rabbit and cat hearts were approximately the same.

On the other hand, the histamine output showed an interesting correlation with the action of digitoxin in rabbit and cat. In these two species there was a significant decrease in the histamine content of the perfusate, which in many cases reached zero in the last sample. However, the histamine output of the rat heart was not affected (Tables III and IV).

It is of interest to note that the time elapsing before complete arrest of auricles and ventricles of the isolated hearts varied with the species. The most resistant was

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[11] Burn, J. H., *Biological Standardization*, 1937, Oxford University Press, London, p. 29.

TABLES V AND VI.
Comparison of the Sensitivity of Rabbit, Cat, and Rat Heart to Perfusion with Digitoxin, 1:40,000.

TABLE V.

| | No. of exper. animal | Amt of digitoxin (1:40,000) ml/g heart to cause heart arrest | Survival time in min. |
|---|---|---|---|
| Rabbits | 16 | 11.5 | 18.0 |
| Avg heart wt equals 9.4 g | 17 | 15.9 | 11.0 |
| | 18 | 13.2 | 12.5 |
| | 19 | 17.0 | 10.3 |
| | 20 | 20.2 | 11.0 |
| | 21 | 12.5 | 7.0 |
| | 23 | — | 11.0 |
| | 24 | 12.4 | 11.0 |
| | | M = 15.9* | M = 11.5* |
| Cats | 1 | 11.4 | 10.5 |
| Avg heart wt equals 14.9 g | 2 | 21.7 | 16.0 |
| | 3 | 21.5 | 13.0 |
| | 4 | 11.3 | 7.0 |
| | 5 | 26.3 | 13.8 |
| | 8 | 29.6 | 10.0 |
| | 10 | — | 15.0 |
| | 11 | 23.6 | 13.0 |
| | 12 | 13.5 | 8.5 |
| | | M = 19.8* | M = 11.9* |

* M = Mean values.

TABLE VI.
Rats—All hearts beating well at one hour after the perfusion started. Average heart weight equals 1.45 g.

| No. of exper. animal | Amt of digitoxin perfused in one hour |
|---|---|
| 1 | 42.0 ml g/hour |
| 2 | 126.2 ,, |
| 5 | 87.1 ,, |
| 7 | 153.8 ,, |
| 8 | 100.0 ,, |
| | M = 101.8* ml per gram per hour |

* M = Mean values.

the rat heart, while the rabbit and cat showed essentially similar sensitivity (Tables V and VI).

*Discussion.* From these results, it is reasonable to assume that the toxic effect of digitoxin on the isolated heart results in decreased histamine output, while the appearance of creatinine in the perfusion fluid is not altered. It has been shown by Anrep and coworkers[6] that when the heart fails the histamine output diminishes. Our results with digitoxin-poisoned hearts corroborate these observations. It is interesting that the relative sensitivities of the 3 species to digitoxin are parallel to their relative sensitivities to histamine. The biochemical mechanism of these findings is being investigated in further detail in order to gain a better understanding of these phenomena.

*Summary.* The perfusion fluid from isolated hearts of cats, rabbits and rats contains creatinine and histamine. There is a species variation in the excretion of these substances. When digitoxin (1:40,000) is added to the perfusion fluid, the creatinine output of the heart is not significantly altered in the 3 species studied. However, the histamine output of the cat and the rabbit heart decreases, while that of the rat heart remains unchanged. The isolated rat heart is much more resistant toward digitoxin than the cat and rabbit hearts.

## 16230

# Experimental Leptospirosis Infection in Chickens.*

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While the number of mammals found to be leptospiral carriers is steadily mounting, nothing is known about such infections in domestic fowl or other birds. Cases of leptospirosis have been reported among personnel of poultry farms and among poultry dressers but rats have always been considered the source of infection, and the possibility that chickens might be infected from rats seems to have been neglected. It seemed of interest, therefore, to examine the susceptibility of chickens to experimental leptospiral infection.

Since no virulent strain of *Leptospira ictero haemorrhagiae* was available, a bovin strain of leptospira,[1] which causes severe infections in cattle and human beings, was used. A heavy growth of the organism on semisolid medium was diluted 1:3 in saline and 1 cc was inoculated subcutaneously into 32 chickens. The birds varied in age from 6 weeks to 5 months.

Twelve birds were bled from the heart daily for 10 days after inoculation and 0.2 to 0.5 cc of blood was inoculated into semisolid or Schueffner's medium or both. Cultures were incubated at 30°C and were examined weekly with the dark field microscope. Positive cultures were obtained from 2 birds, one on the fourth and one on the ninth day after injection. This low proportion of positive cultures may have been due to the fact that chicken serum when added to heavily inoculated Schueffner medium has a harmful effect on the organism and inhibits growth.

The bird with the positive blood culture on the 9th day showed no clinical signs of illness but serum taken at 3 weeks had an agglutinin titer of 1:800. When the animal was sacrificed at 4 weeks no pathological changes were found and no organisms were seen in sections of organs stained by Levaditi's method. A calf which was injected with 1 cc of a culture of the isolated strain reacted with a short fever (41.5°C) and developed an agglutinin titer of 1:1600 against the bovine strain of leptospira.

The bird with the positive culture on the fourth day became sick on the 8th day and was sacrificed. Liver, spleen and kidney were cultured on semisolid medium and organisms were successfully cultivated from the liver where they were also demonstrated in considerable number in sections stained by Levaditi's method. The organisms were found within well preserved liver cells. In some areas the liver cells were detached from one another and numerous leucocytes were found in the capillaries. A few organisms were found in sections of the kidney but they could not be cultivated from this organ or the spleen. There were no gross pathological changes on autopsy.

Five chickens were found dead between the 9th and the 14th day after inoculation and 5 more were sacrificed between the 6th and 9th day. No specific changes were found in these birds on autopsy. No leptospiras were demonstrable with certainty in Levaditi stained organ sections although argentophile coils of undetermined nature were seen a number of times.

All of the surviving birds were bled at one to 2 week intervals for serological tests. Sera in dilutions of 1:20 and 1:200 were tested for the agglutination of formolized suspensions of *L. icterohaemorrhagiae*† and the bovine leptospiral strain. Equal volumes of serum dilution and antigen were mixed in small test tubes and incubated at 37°C for 2 hours. The results were read the following

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* This work was aided by a grant from the Palestine Board for Scientific and Industrial Research.

[1] Bernkopf, H., Olitzki, L., and Stuczynski, L. A., *J. Infect. Dis.*, 1947, **80**, 53.

† The Jackson strain kindly supplied by Dr. J. C. Broom, The Wellcome Laboratories of Tropical Medicine, London.

morning by examining uncovered drops with a low power objective with dark field illumination. All the pre-inoculation bleedings were negative and the antibody response was very variable. Of six, 6-week-old birds, 3 developed no agglutinins at all and 3 developed titers of only 1:40. Among the 2- to 5-month-old birds, levels of 1:800 to 1:8000 were reached and during a 2- to 3-month period of observation these titers did not fall appreciably.

Since the successful demonstration of infection in subcutaneously inoculated birds was demonstrated in only 2 instances the actual number of infections accomplished cannot be stated since the antibody response in older birds may have resulted from the antigenic stimulus of the inoculum. This possibility was eliminated in another experiment in which 5 birds were given the organism by feeding. Each bird received 1 cc of a fluid leptospiral culture drop by drop with a pipette, care being taken that the material was slowly swallowed and that the mucous membranes were not traumatized. Three weeks later all 5 birds had developed specific serum antibodies in titers ranging from 1:800 to 1:3200. This established the susceptibility of domestic fowls to infection in a manner which could well occur in nature. The actual occurrence of infection in nature remains to be demonstrated.

More than 500 chicken sera, from several flocks, have been examined for their agglutinin titer with bovine leptospira and *L. icterohaemorrhagiae* and while titers of 1:20 and 1:40 were frequently observed these are not considered significant. Two sera were found with titers of 1:400 with both strains and one serum which was positive at 1:200 with the bovine strain only. These serological findings though not adequate in themselves, suggest the occurrence of leptospira infections in chickens in nature.

*Summary.* Domestic chickens were shown to be suceptible to experimental infection with a bovine strain of leptospira, when given subcutaneously or by mouth. The infection occurs in inapparent form, as a rule.

### 16231

## Influence of Fluorine in Mottled Teeth on Dental Caries.*

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If compounds of fluorine are to be used as drugs in combating human dental caries,[1] eventually the manner of action must be explained in order to direct their most practical application. At present the literature assumes that it works (1) through a constant intake of fluorine-bearing water[2] or (2) by direct chemical combination with the constituents of the tooth, introduced either prenatally[3] or postnatally.[4] The latter assumption has been contested by the presentation of data which shows that a significant effect of fluoride acquired after tooth eruption on induced rat caries is not evident.[5] Using

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* The investigation upon which this report is based was made possible by funds granted by the Carnegie Corporation of New York to the Division of Dental Research, School of Medicine and Dentistry, The University of Rochester, Rochester, N.Y.

[1] Editorials, *J. A. D. A.*, 1947, **34**, 345; *J. A. D. A.*, 1947, **34**, 411.

[2] Dean, H. T., *Am. J. Ortho. and Oral Surg.*, 1947, **33**, 49.

[3] Cox, G. J., Matuschak, M. C., Dixon, S. F., Dodds, M. L., and Walker, W. E., *J. D. Res.*, 1939, **18**, 481.

[4] Armstrong, W. D., and Brekhus, P. J., *J. D. Res.*, 1938, **17**, 393.

[5] McClure, F. J., *J. Nutrition*, 1941, **22**, 391.

TABLE I.

| Group | Condition of animal | No. of animals | No. of molar teeth examined | No. fractured cusps per animal | No. carious cuspal involvements per animal | No. carious teeth per animal |
|---|---|---|---|---|---|---|
| I | Normal | 15 | 180 | 10.9 | 4.0 | 1.8 |
| II | Extirpated salivary glands | 12 | 144 | 4.9 | 36.6 | 10.6 |
| III | Mottled molars | 9 | 108 | 20.0 | 5.4 | 1.5 |
| IV | Extirpated salivary glands and mottled molars | 9 | 108 | 12.6 | 28.1 | 8.5 |

a technique for desalivating rats worked out in our laboratory,[6] it has been possible to demonstrate that a continuous supply of fluorine will arrest caries in the absence of saliva.[7] The present report is based upon further studies with desalivated animals. Using the technique in combination with a method for increasing the fluorine content of rat molars, also worked out in our laboratory,[8] it has been possible to remove the influence of saliva in animals previously subjected to fluorine in sufficient amounts to cause mottling during prenatal life and test the influence of the fluorinized tooth acting alone against caries-producing conditions.

*Experiments and Results.* A total of 18 rats were obtained from three mothers which had been receiving daily concentrations of 300 to 350 parts per million of fluorine as potassium fluoride during pregnancy, and a total of 27 young were selected from five mothers which received no fluorine. All parents were from the same Wistar breeding stock. One-half of the fluorinized young and 12 of the 27 nonfluorinized young were subjected to total salivary gland extirpations at the age of 24 days. On the 42nd day all animals were placed on a caries-producing diet.[9] The food and distilled water were given *ad lib.* Weights were taken every 4 days. After 104 days on the diet, the animals were chloroformed, the jaws were separated, and all molar teeth examined with a sharp explorer tine under a binocular microscope, magnification 30X. Several sagittal planes of jaw segments were examined histologically in ground and decalcified section to confirm the accuracy of the diagnosis. Lesions were classified into simple fractures and carious cavities. Presumably mechanical fracture of rat molars precedes most experimental carious destruction. Male and female differences were not sufficiently significant to warrant separate consideration in any group.

Observation in Table I shows that the reduction of saliva greatly increases the incidence of rat caries. This also has been demonstrated in a previous publication by us.[10] Normal animals on the caries-producing diet average 4.0 carious cuspal involvements and 1.8 carious teeth per animal; those with salivary glands extirpated average 36.6 carious cuspal involvements and 10.6 carious teeth per animal; those with mottled molars average 5.4 carious cuspal involvements and 1.5 carious teeth per animal; and desalivated rats with mottled molars average 28.1 carious cuspal involvements and 8.5 carious teeth per animal. The number of cavities in the lower teeth exceeded those in the upper for all animals examined. Because the average number of carious teeth in the third group is only slightly lower than normal it seems that the fluorine incorporated in the tooth substance has very little, if any, influence in retarding caries. The greater number of carious cuspal involvements in the same group gives added support to this assumption. The outstanding conclusions, however, are based upon a comparison of the last group with the remaining three. Desalivated animals with mottled molars show an increase of approximately 80% of carious lesions over normal animals and only about 20% reduction compared to desalivated animals not subjected to fluorine in their prenatal period. The relatively low number of fractures in nonfluorinized animals subjected to desalivation suggests that early breaks rapidly become carious. The higher number of fractures in the normal animals indicates the reverse condition, but in the fluorinized animals of Groups 3 and 4 it would be difficult to say to what degree the high frequency of breaks was due to greater friability of hypoplastic fluorinized teeth.

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[6] Cheyne, V. D., *J. D. Res.*, 1939, **18**, 457.

[7] Cheyne, V. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 58.

[8] Cheyne, V. D., *J. D. Res.*, 1942, **21**, 145.

[9] Hoppert, C. A., Webber, P. A., and Canniff, T. L., *J. D. Res.*, 1932, **12**, 161.

[10] Cheyne, V. D., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 587.

*Summary.* Some of the factors operative in suppressing rat caries have been tested in this experiment. By using animals in which the salivary glands have been removed it has been possible to test the relative effects of fluorine prenatally incorporated in the teeth. When the caries score of operated rats with mottled or fluorinized teeth are compared with that of nonfluorinized animals without glands there is a measurable reduction in caries. When the percentage of reduction is compared with the caries score of normal animals, however, there is evidence that the saliva offers greater protection than the fluorine in tooth substance.

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### 16232

## Prevention of the Anti-Curare Action of Epinephrine by Dibenamine.

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The effects of N, N-dibenzyl-beta-chloroethylamine (Dibenamine) on the responses of various visceral effectors to epinephrine have been studied by Nickerson and Goodman.[1] They have reported adrenolytic actions of Dibenamine in various visceral effectors which have an excitatory adrenergic innervation. Epinephrine is known to exert an anti-curare action at the skeletal neuromuscular junction.[2,3] The mechanism of this action is unknown. It does not appear to be related to any influence on adrenergic neuro-effector systems. In the present study it is demonstrated that Dibenamine prevents the anti-curare action of epinephrine.

*Methods.* Seven dogs were used. Intravenous sodium pentobarbital was the anesthetic employed in 6 dogs. In one animal, intratracheal ether anesthesia was used following the administration of 15.0 mg morphine sulfate. The peripheral portion of the cut peroneal nerve was drawn across 2 glass-shielded platinum electrodes, and single shocks of supramaximal strength were applied every 2 seconds by means of an electronic stimulator (Electrodyne, model 461). Relatively isometric contractions of the tibialis anticus muscle were recorded on a revolving drum by means of a Meyerhof type of myograph. The femoral artery of the same side was exposed for intra-arterial injections. The femoral vein of the opposite side was exposed for intravenous injection.

The curare preparation used was Intocostrin, 20 units per cc (E. R. Squibb and Sons). This preparation has the same potency as a solution containing 2.7 mg of anhydrous d-tubocurarine chloride per cc. The following general procedure was used in each of the 7 experiments. Intocostrin was diluted with isotonic saline so that each cc contained 2 units, and this was injected intravenously in amounts sufficient to depress the amplitude of muscular contraction to between one-fourth and one-half of the original height. Then epinephrine (Adrenalin, Parke-Davis and Co.) was injected rapidly intra-arterially. After demonstrating the anti-curare action of

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[1] Nickerson, M., and Goodman, L., *J. Pharm. and Exp. Therap.*, 1947, **89**, 167.

[2] Rosenblueth, A., Lindsley, D. B., and Morison, R. S., *Am. J. Physiol.*, 1936, **115**, 53.

[3] Wilson, A. T., and Wright, S., *Quart. J. Exp. Physiol.*, 1936-37, **26**, 127.

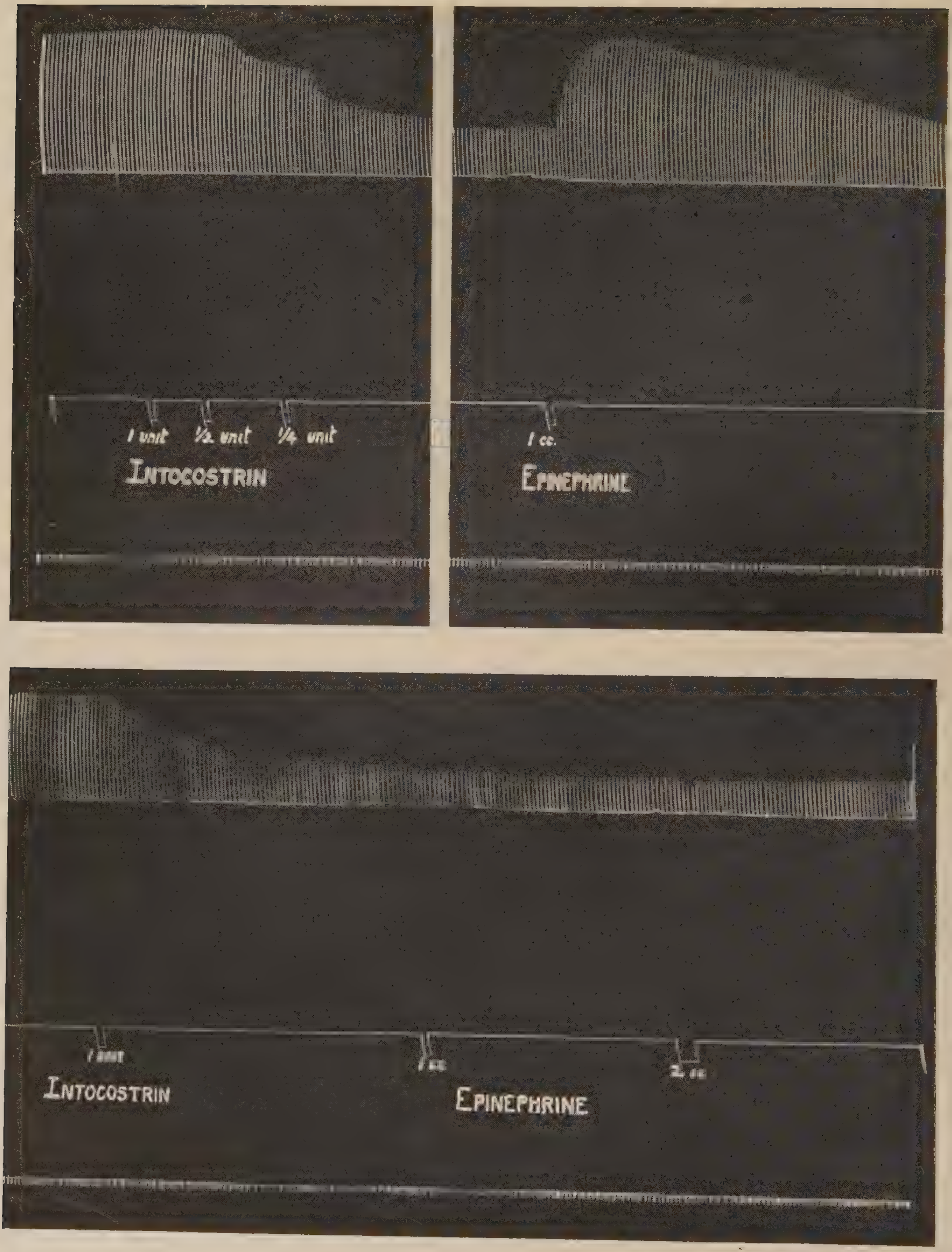

FIG. 1.

Prevention of the Anti-curare Action of Epinephrine by Dibenamine.

Upper Record. *Before Dibenamine.* From above downward record shows: (1) contractions of tibialis anticus muscle produced by stimulating the peroneal nerve at 2-second intervals. (2) times of intravenous injection of Intocostrin and intra-arterial injection of epinephrine

(1:100,000), and (3) time in 2-second intervals. At the break in the record a 3-minute section was removed, during which time an additional 0.25 unit of Intocostrin was injected.

Lower Record. *After Dibenamine.* Recording as in upper record. Animal had received 2 units of Intocostrin approximately 1 minute before the injection of Indocostrin indicated on the record.

epinephrine, Dibenamine was administered intravenously. The hydrochloride salt of Dibenamine was dissolved in propylene glycol (20 mg per cc) and then diluted with an equal amount of distilled water. Each dog was given between 25 and 45 mg of Dibenamine per kg within a period of 15 to 60 minutes. Within one to 3 hours after injection of Dibenamine, administration of curare and epinephrine was repeated.

*Results.* Intra-arterial injection of epinephrine (1.0 or 2.0 cc of a 1:100,000 solution of epinephrine in saline), following partial curarization, caused an immediate marked increase in the amplitude of muscular contraction in each of the 7 animals tested. A typical response is illustrated in Fig. 1, upper record.

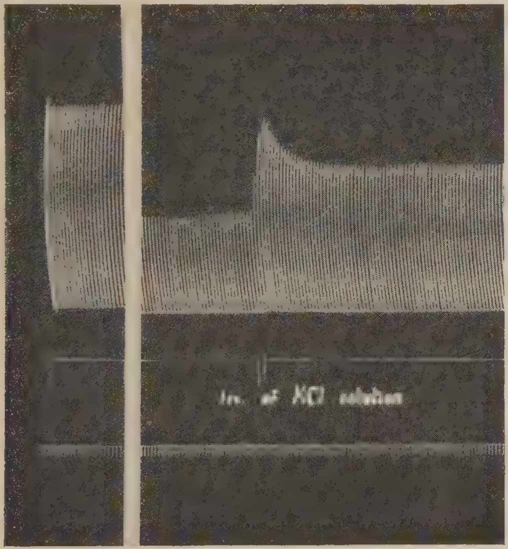

FIG. 2.

Anti-curare Action of Potassium after Dibenamine. Writing points are arranged as in Fig. 1. The record was obtained approximately 2 hours after administration of Dibenamine, 30 mg per kg. The break in the record indicates an 11-minute interval during which 1.2 units of Intocostrin were administered intravenously. Isotonic potassium chloride was administered intra-arterially.

After administration of Dibenamine, and subsequent partial curarization, the same dose of epinephrine failed to increase the amplitude of contraction in each of the 7 animals. An experiment is illustrated in Fig. 1, lower record. In fact, the injection of the epinephrine solution after Dibenamine and partial curarization produced a slight transient decrease in the amplitude of the contractions. The following controls indicate that the decreased amplitude was not due to the epinephrine but was related to the volume of the diluent. 1) In each of 2 dogs tested, injection of the same amount of epinephrine (20 $\mu$g) in 0.02 cc of fluid (*i.e.* 0.02 cc of a 1:1,000 solution of epinephrine) failed to have any effect on the amplitude of contraction. 2) The same slight depression in amplitude of contraction was produced in 3 dogs by the intra-arterial injection of 1.0 to 2.0 cc of isotonic saline or water.

The effects of large doses of epinephrine were tested in three dogs. A dose of epinephrine 100 times that used to demonstrate anti-curare action before Dibenamine had no anti-curare effect after Dibenamine.

Isotonic potassium chloride solution (1.15%), 1.0 to 5.0 cc was administered intra-arterially to 3 dogs following partial curarization. The well-known immediate increase in amplitude of contraction was noted both before and after Dibenamine. The anti-curare action of potassium chloride following the administration of Dibenamine is illustrated in Fig. 2.

*Summary.* The effects of Dibenamine on the anti-curare actions of epinephrine and of KCl have been studied in 7 dogs anesthetized with sodium pentobarbital or with morphine and ether. Dibenamine, in doses of 25-45 mg per kg, prevents the anti-curare action of epinephrine but does not prevent the anti-curare action of potassium chloride.

## 16233

# Alloxan Diabetes in Mice.

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Alloxan diabetes has been produced in rabbits, rats, dogs, cats, monkeys, pigeons, and guinea pigs.[1-5] Although Dunn *et al.*,[6] state that alloxan causes islet lesions in mice, no mention was found in the literature of the production of alloxan hyperglycemia in this species. In the course of studies on the effect of hyperglycemia on susceptibility to bacterial infection, it became desirable to find a consistent means of inducing this condition in a small laboratory animal such as the mouse.

*Materials and Methods.* Male Swiss albino mice of the same age and weight were selected for each experiment. A fresh aqueous solution of alloxan monohydrate (4 mg/ml for intravenous injection and 6 mg/ml for intraperitoneal and subcutaneous injections) was used. Blood sugars were determined by the method of Reinecke.[¶,7] Animals with blood sugar levels over 200 mg% were considered hyperglycemic, since blood sugar determinations on 20 control mice on food and water *ad libitum* were all under 150 mg%.

In view of the wide variation of dosage and route of administration necessary to produce alloxan diabetes in different species of animals,[1-5,8] the following procedures were carried out.

a. Intravenous tail injections of doses varying from 50 mg/kilo to 300 mg/kilo of alloxan were given to 65 mice maintained on Purina Mouse Chow and water *ad libitum* before and after injection. Blood sugars were determined 48 hours after inoculation. Intraperitoneal and subcutaneous injections of similar dosages of alloxan were given to additional groups of mice on the same diet.

b. Since it has been demonstrated[8] that fasting prior to injection increases the susceptibility of rats to alloxan, 131 mice fasted for 8 to 24 hours, were given alloxan intraperitoneally and fed Purina Mouse Chow immediately thereafter.

c. Hard[9] reported that some animals of a given species are resistant to repeated injections of alloxan. A number of mice previously unaffected by an initial dose of alloxan were therefore reinjected with similar dosages and later tested for hyperglycemia.

d. In order to determine the duration of alloxan hyperglycemia blood sugar determinations were made on groups of diabetic mice at various times after the injection of alloxan.

e. Inasmuch as riboflavin, epinephrine, glutathione, cysteine, and niacin have been reported to protect against alloxan[10,8,11,12] an investigation of their protective action in mice was made.

*Results.* The optimal dose of alloxan for the production of hyperglycemia in mice was found to be 100 mg/kilo injected directly into a tail vein. All of 50 mice given alloxan in this dosage were found to be hyperglycemic at the end of 48 hours, while none of the animals died (Table I) (A).

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[*] Technical assistance of James Samsell.

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TABLE I.
The Mortality and Blood Sugar Levels of Mice 48 Hours After Inoculation of Alloxan.

| No. of animals | Conditions prior to inj. of alloxan | Dose of alloxan mg/kilo | Route of injection | No. of deaths 48 hr | Blood sugar levels after 48 hr mg % 50-150 | 150-200 | 200-300 | Greater than 300 |
|---|---|---|---|---|---|---|---|---|
| (A) | | | | | | | | |
| 5 | Food and water *ad lib.* | 300 | Intravenous | 5 | 0 | 0 | 0 | 0 |
| 5 | | 200 | ” | 3 | | | | 2 |
| 50 | | 100 | ” | 0 | 0 | 0 | 0 | 50 |
| 5 | | 50 | ” | 0 | 2 | | | 3 |
| (B) | | | | | | | | |
| 18 | Same | 400 | Intraper. | 3 | 4 | 3 | 4 | 4 |
| 195 | | 300 | ” | 19 | 23 | 52 | 37 | 64 |
| 8 | | 200 | ” | 0 | 3 | 3 | 2 | 0 |
| 8 | | 100 | ” | 0 | 4 | 2 | 1 | 1 |
| (C) | | | | | | | | |
| 6 | Same | 750 | Subcut. | 2 | 2 | 0 | 0 | 2 |
| 6 | | 600 | ” | 4 | 1 | 0 | 0 | 1 |
| 6 | | 500 | ” | 2 | 2 | 1 | 0 | 1 |
| 6 | | 400 | ” | 3 | 3 | 0 | 0 | 0 |
| 6 | | 300 | ” | 0 | 6 | 0 | 0 | 0 |
| 6 | | 200 | ” | 0 | 1 | 4 | 0 | 1 |
| 6 | | 100 | ” | 0 | 4 | 1 | 1 | 0 |
| (D) | | | | | | | | |
| 10 | Fasted 24 hr | 400 | Intraper. | 3 | 0 | 0 | 1 | 6 |
| 12 | ” ” ” | 300 | ” | 0 | 0 | 0 | 0 | 12* |
| 100 | ” 8 ” | 300 | ” | 45 | 2 | 6 | 15 | 32 |
| 9 | ” 24 ” | 300 | Subcut. | 0 | 4 | 2 | 3 | 0 |

* Five of these mice died after 60 hours.

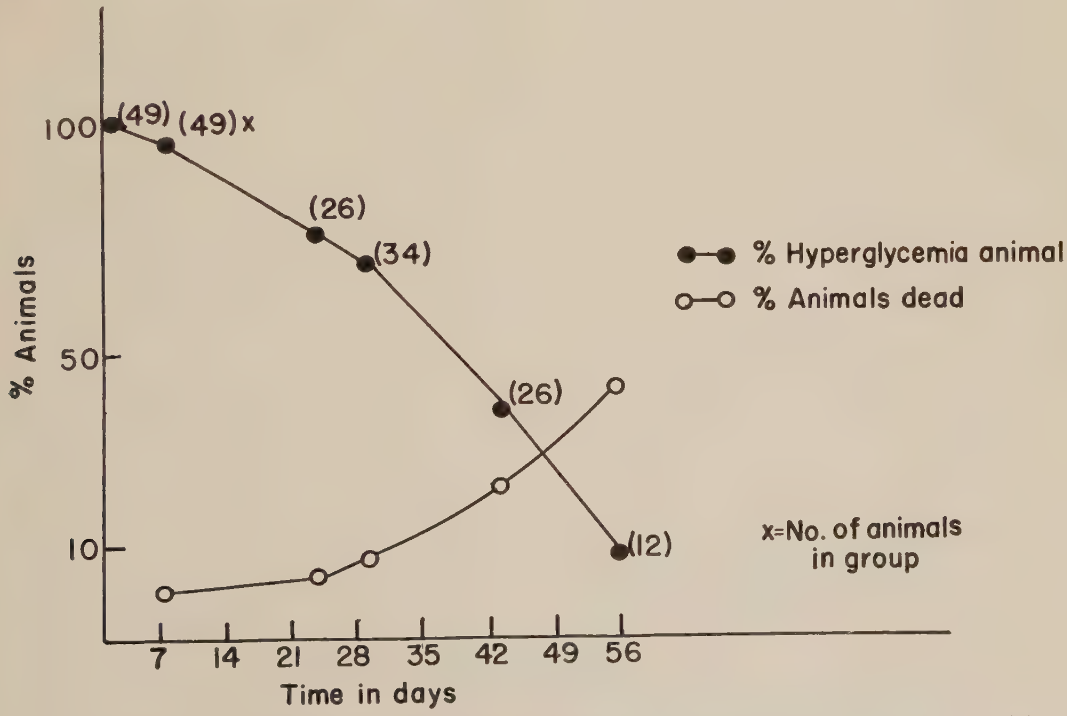

Figure I.— Duration of hyperglycemia and mortality after inoculation with alloxan

TABLE II.

Effect of Riboflavin, Epinephrine, Cysteine, and Niacin on Development of Alloxan Hyperglycemia in Mice.

| No. of animals | Compound tested | Route of inj. | Time given before inj. of alloxan* | Dose of compound | Death | Blood sugar levels after 48 hr, mg % | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 50-150 | 150-200 | 200-300 | Over 300 |
| 20 | Riboflavin | Intraper. | 6 hr | 50 mg | 16 | 1 | 0 | 2 | 1 |
| 20 | ,, | ,, | 6 ,, | 10 ,, | 3 | 5 | 7 | 1 | 4 |
| 20 | ,, | Subcut. | 6 ,, | 10 ,, | 0 | 1 | 3 | 1 | 15 |
| 60 | Epinephrine | ,, | 3 min. | .1 cc, 1:10,000 | 10 | 13 | 8 | 10 | 19 |
| 10 | Glutathione | Intraper. | 30 ,, | 40 mg | 8 | 2 | 0 | 0 | 0 |
| 20 | Cysteine | ,, | 1 hr | 5 ,, | 20† | 0 | 0 | 0 | 0 |
| 20 | ,, | Subcut. | 30 min. | 3 ,, | 8 | 6 | 3 | 4 | 2 |
| 20 | ,, | ,, | 1 hr | 1 ,, | 4 | 0 | 4 | 3 | 9 |
| 20 | Niacin | ,, | 6 ,, | 5 ,, | 4 | 5 | 1 | 6 | 4 |
| 195 | Control | — | — | — | 19 | 23 | 52 | 37 | 64 |

* All mice were given 300 mg/kilo of alloxan intraperitoneally.
† All mice died before injection of alloxan.

The optimal dose for the intraperitoneal injection of alloxan was found to be 300 mg/kilo. Of 195 mice given this dose, 101 (52%) showed a hyperglycemia greater than 200 mg% in 48 hours (Table I) (B). Nineteen of the 195 (9%) mice died in 48 hours. Alloxan given in similar dosages by the subcutaneous route was found to be less effective in that only 6 of 42 mice so treated developed hyperglycemia (Table I) (C).

Fasting for 8 to 24 hours prior to injection increases the mortality due to alloxan without increasing the incidence of hyperglycemia in the survivors. Although only 60 of 131 (45%) fasted animals were hyperglycemic 48 hours after injection, 48 mice (37%) died in this interval (Table I) (D).

Five of 22 mice demonstrated to be resistant to an initial dose of alloxan were found to be hyperglycemic after a second injection of the same dose.

The diabetes produced by alloxan was found to be relatively transient in that at the end of 6 weeks 20 or 26 initially diabetic mice had survived and of these only 10 remained hyperglycemic (Fig. 1).

In the dosages used epinephrine, cysteine, niacin and riboflavin gave no significant protection against the action of alloxan (Table II).

*Summary.* The most effective dose and route of administration of alloxan were found to be 100 mg/kilo injected into the tail vein of the mouse. Fifty mice treated in this way all had blood sugar levels greater than 300 mg% 48 hours later. There were no mortalities.

Fasting before the intraperitoneal injection of alloxan increased the mortality in mice without increasing the incidence of diabetes among the survivors.

Mice resistant to the first injection of alloxan were not necessarily resistant to a second injection of the same dose.

Alloxan hyperglycemia was not permanent in all mice, since the blood sugar levels of one-half of the survivors reverted to normal within 6 weeks.

In the dosages administered epinephrine, cysteine, niacin and riboflavin did not give protection against the action of alloxan.

16234

# Tests of Possible Antagonism of Gold for Histamine Toxicity and Certain Allergic Reactions.*

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There has been considerable discussion about the possible allergic nature of rheumatoid arthritis as a disease process. The supporting evidence has been both clinical and experimental. According to Hench,[1] "no one has been able to set up any one particular syndrome as allergic arthritis or to prove that any of the arthritides are chiefly or solely the results of bacterial or food allergy." However, many attempts have been made to determine the possible role of bacterial allergy in the production of rheumatoid arthritis.[2] Klinge has reported the production of an arthritis in rabbits sensitized to horse serum by subsequently injecting horse serum into the joints.[3] More recently Rich and Gregory have suggested that the characteristics of rheumatic carditis develop as a result of anaphylactic hypersensitivity.[4]

At the same time, increasing evidence has accumulated that gold salts favorably influence the clinical course of rheumatoid arthritis.[5,6,7] In rats and mice the experimental arthritis produced by the pleuropneumonia-like organisms is prevented by the use of gold salts and the disease once begun is arrested by their use.[8,9]

In view of the favorable claims for an allergic factor in rheumatoid arthritis and because gold salts seem to alter favorably the course of the disease in some unknown way it was thought desirable to determine whether the gold ion could prevent histamine toxicity and anaphylactic shock and beneficially effect the Arthus phenomenon in animals. If it could be demonstrated that the gold ion altered beneficially experimental allergic reactions, the mode of beneficial action of gold in rheumatoid arthritis would be indicated, and conversely, if such experimental responses were not altered, this would suggest that gold acted in some other manner.

*Anti-Anaphylactic Effect.* Ten guinea pigs (weighing from 210 to 375 g) were each given 0.1 cc of horse serum hypodermically, and on the same day and every 3 days thereafter for 7 doses the guinea pigs were given 10 mg per kg of gold sodium thiosulfate intramuscularly. Twenty-eight days after the initial injection of the horse serum the animals were given 0.5 cc of horse serum intravenously and 11 of the 12 animals died in 5 to 15 minutes. Twelve control animals were given similar doses of horse serum without gold sodium thiosulfate, and only 1 animal survived in this group.

*Acute Histamine Toxicity.* Ten guinea pigs (weighing from 210 to 375 g) were given 10 mg per kg of gold sodium thiosulfate intramuscularly for 5 doses in a 7-day period and 4 of the animals died during the week. At the end of the week the 6 remaining animals, which had received gold were given 1 mg per kg of histamine phosphate intravenously, and they all died in 1 to 5

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* This work was done under contract with the Office of Naval Research.

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minutes. Nine guinea pigs in the same weight range were taken as controls and given only the histamine phosphate in the same dosage, and they all died in 1 to 5 minutes after the injection.

*Arthus Phenomenon.* Rabbits were used to determine the possible ameliorating effects of gold sodium thiosulfate on the Arthus phenomenon. The method was as follows: 10 rabbits were sensitized by weekly subcutaneous injections of 1 cc of a 1 to 10 dilution of horse serum in physiological saline solution, according to the method previously used in this department.[10] After sensitization was established, as shown by swelling at the site of injection, the rabbits were divided into 2 groups of 5 each. One group was given weekly intravenous injections of gold sodium thiosulfate beginning at 10 mg per kg and increasing to 30 mg per kg. The total dosage was 100 mg per kg in 6 weeks. The other group of 5 rabbits were kept as a control. The day following the intravenous injections of gold sodium thiosulfate, all 10 animals were given a subcutaneous injection of 1.0 cc of a 1 to 10 dilution of horse serum in physiological saline. The diameters of the resulting swellings were recorded at the end of 24 hours. The average diameters of the swellings in the control group ranged from 2.5 to 4.0 cm and in the gold treated group from 2.4 to 4.6 cm. Thus there was no demonstrable difference between the treated and untreated animals in the size of the wheals resulting from the horse serum.

Gold was given intravenously to these rabbits as a matter of convenience and because this method is also used clinically.

*Discussion.* Evidently gold sodium thiosulfate given intramuscularly to guinea pigs offers no protection against anaphylactic shock, or the acute toxicity of histamine. Further, gold sodium thiosulfate does not affect the development of the characteristic wheal in the Arthus phenomenon; although, it should be stated that the antihistamine agents, pyribenzamine and benadryl, likewise fail to inhibit the development of this phenomenon.[11] However, Kyser, McCarter, and Stengle have recently reported that antihistamine drugs impede the development of myocardial lesions in rabbits injected with horse serum.[12] Since gold sodium thiosulfate does not appear to alter these manifestations of experimental sensitivity, it seems reasonable to assume that the beneficial action of gold therapeutically also does not rest on inhibition or suppression of allergic responses.

*Conclusions.* (1) Gold sodium thiosulfate failed to protect guinea pigs against lethal doses of histamine and anaphylactic shock, and did not demonstrably alter the magnitude of the Arthus phenomenon in rabbits. (2) These negative results suggest that the gold ion mediates its beneficial effects in rheumatoid arthritis in ways other than by amelioration of objective allergic manifestations.

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16235

# Chemotherapy of Tuberculosis III. *In Vitro* and *in Vivo* Activities of Various Compounds.

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Continuing our studies of antituberculosis agents we have examined a heterogeneous group of compounds *in vitro,* and in addition, the sodium formaldehyde bisulfite addition product of 2-butoxy-5-aminopyridine has been evaluated *in vivo.* The results of these studies are set forth in the experimental part.

*Experiments in vitro.* The compounds, listed in Table I, were dissolved in Dubos' medium,[1] using the two-fold serial dilution method. The starting concentration was 64 mg%, or if the substance was not soluble to that extent, a saturated solution was used. Controls were included in every series, and each experiment was run in duplicate. The organisms were the pathogenic tubercle bacilli, H37RV and B1. The standard inoculum was 0.1 ml of a 14 day culture in Dubos' medium which had been diluted to an optical density of 0.2 as measured in the Lumetron colorimeter. Two weeks after inoculation tubes were examined for growth, and when readings were doubtful Ziehl-Neelsen stained smears were examined.

It is apparent from Table I, that none of the chemicals listed, with the exception of the p-aminosalicylic acid preparations, possess any great activity *in vitro* against tubercle bacilli. The differences in bacteriostatic activity between the different samples of p-aminosalicylic acid undoubtedly may be ascribed to differences in the purity of the samples. It should be noted that the findings with Compound 13 are completely different from those previously reported.[2] The differences in activities of Compound 13, and p-aminosalicylic acid from those previously reported[2,3,4] are almost certainly due to important differences in technique. Compounds numbered 6, 7, 8, and 14 are being further studied. Compound 19, a commercial product sold under the name "Mandelamine," has important antibacterial properties[5,6] apart from its antimycobacterial activity. Its effect on experimental tuberculosis is now under study.

*Experiments in vivo.* Mice of the highly susceptible[7] dba strain, average weight 20 g, were injected intravenously with 0.1 ml of a suspension containing 0.1 mg of *My. tuberculosis,* strain B1. One week after infection, the animals were divided into 3 groups. One group was given Compound 13 mixed into the ground diet in 2% concentration. A small group of mice was treated similarly, for comparative purposes, with p-aminosalicylic acid, the therapeutic effect of which is well known.[3,4,8] The third group of mice remained untreated. The food cups were weighed daily and the amount of drug which each mouse ingested was estimated. The mice were weighed twice weekly. Surviving animals were sacrificed 32 days after infection. At autopsy the disease present in the lungs, liver, spleen, and kidneys was graded according to its extent and character. The scores for all organs were averaged and the average score, graded from 0 to 4+, was calculated for each group. A 4+ rating indicates caseous progressive tuberculosis of more than 50% of each organ.

The results of these experiments are given in Table II. While p-aminosalicylic acid

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[1] Dubos, R. J., and Davis, B. D., *J. Exp. Med.,* 1946, **83**, 409.

[2] Feinstone, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 153.

[3] Lehmann, J., *Lancet,* 1946, **1**, 15.

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TABLE I.
Lowest Bacteriostatic Concentrations mg %* of the Various Compounds Which Inhibit the Growth of Strains of Pathogenic Mycobacteria. Dubos' medium, inoculum 0.1 ml, incubation time, 14 days.

| Number | Compound | H37RV | B1 |
|---|---|---|---|
| 1 | *p*-aminosalicylic acid HCl | 0.125 | 0.125 |
| 2 | " " " Lehmann† | 1.0 | 1.0 |
| 3 | " " " Youmans‡ | 1.0 | 1.0 |
| 4 | *p*-phenetidin HCl | 16. | 32. |
| 5 | acetophenetidin | >32. | >32. |
| 6 | *p*-acetamidobutoxybenzene | 8. | 16. |
| 7 | diethylaminoethoxy-2,4,5-trichlorobenzene HCl | 8. | — |
| 8 | " -2-naphthalene HCl | 8. | 16. |
| 9 | 3-hydroxy-4-methylpyridine | >64. | >64. |
| 10 | " " -N-oxide | >64. | >64. |
| 11 | " -4-methyl,1-2′,3′-dihydroxypropyl-pyridinium chloride | >64. | >64. |
| 12 | 2-methoxy-5-aminopyridine dihydrochloride | 32. | >64. |
| 13 | 2-butoxy-5- " sodium formaldehyde bisulfite | >64. | >64. |
| 14 | 2-butoxy-5-acetamidopyridine | 8. | 32. |
| 15 | 2′,6′-diamino-2-butoxy-5,5′-azopyridine | >4. | > 4. |
| 16 | 2-amino-6-hydroxypyridyl-3,4′-azo-1′-hydroxybenzene | >4. | > 4. |
| 17 | 2-amino-6-hydroxypyridyl-3,4′-azo-1′-hydroxybenzene | >4. | > 4. |
| 18 | p,p′-di(4-hydroxy-3-carboxy-phenylazo) diphenyl sulfone tetrasodium salt | — | 32. |
| 19 | methenamine mandelate | 16. | 16. |

* Numbers preceded by the symbol > indicate the concentrations of saturated solutions.

† Obtained through the kindness of Professor Jorgen Lehmann, Sahlgrenska Sjukhuset, Gothenburg, Sweden.

‡ Obtained through the kindness of Dr. Guy P. Youmans, Northwestern University Medical School, Chicago, Ill.

TABLE II.
Results of Treating Tuberculous dba Mice with *p*-Aminosalicylic Acid (No. 1), and with 2-Butoxy-5-aminopyridine Sodium Formaldehyde Bisulfite (No. 13).

| Compound No. | Drug conc. in diet | No. mice | Avg amt drug ingested g/kg/day | No. survivors | Amt tuberculosis |
|---|---|---|---|---|---|
| 0 | — | 15 | — | 15 | 2+ |
| 13 | 2% | 15 | 4.5 | 4 | 3+ |
| 1 | 2% | 9 | 3.9 | 9 | 1+ |

exerts a favorable influence on tuberculous dba mice, Compound 13 appears to exert deleterious effects. Because the amount of Compound 13 ingested by the mice is more than twice its acute $LD_{50}$,[9] it seems probable that the poor results may be in part attributed to toxic effects of the drug.

Guinea pigs were employed in the second *in vivo* test of Compound 13. Eighty-five tuberculin negative guinea pigs, average weight 300 g, were infected subcutaneously in the groin with 0.5 mg (moist weight) B1 per 500 g body weight. Eight days later, when all animals reacted to 1 mg O. T. (Mantoux), they were divided into 5 groups and treated as follows:

Group 1. 20 guinea pigs received Compound 13 in their food in 2% concentration.

Group 1A. 15 guinea pigs, receiving the same treatment as Group 1, were used for blood studies.

Group 2. 20 guinea pigs received Compound 13 subcutaneously, 0.17 g per kg 3 times daily at 9:00 A. M., 5:00 P. M., and 12:00 M.

Group 2A. 15 guinea pigs, receiving the same treatment as Group 2, were used for blood studies.

Group 3. 15 guinea pigs were used as

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[9] Feinstone, W. H., Friedman, H. L., Rothlauf, M. V., Kelly, A. M., and Williams, R. D., *J. Pharm. and Exp. Therap.*, 1947, **89**, 153.

untreated controls.

All animals were weighed and tuberculin tests (Mantoux) with 1 mg O. T. were performed at weekly intervals. Blood, obtained by cardiac puncture from the animals in the subgroups, was used for the estimation of hemoglobin and Compound 13. Hemoglobin was measured in the Lumetron colorimeter and Compound 13 was measured according to the Marshall method.[10] Blood for the latter test was drawn in the morning from the animals of Group 1A, and at specified intervals from animals of Group 2A. Each animal was autopsied as soon as possible after death. Surviving animals were sacrificed 90 days after infection. Upon autopsy the amount of tuberculosis involvement in the lungs, liver, spleen, and the lymphatic system was expressed in values proportional to the extent and severity of the disease. As in the preceding experiment, a rating of 4+ indicates that the organ exhibited widespread caseous lesions. The average ratings for the various organs represent a summary of the tuberculosis present in the animal. Representative blocks of tissue were sectioned and examined microscopically after staining with hematoxylin-eosin and with Ziehl-Neelsen stains.

Upon treating the tuberculous guinea pigs with the drug it was found that subcutaneous administration of the drug caused the appearance of broad shallow ulcers at the site of injection. These developed 2 to 3 days after treatment was started, were about 1 cm in diameter, and sometimes penetrated to the underlying muscle. Six or 7 such ulcers were present in most of the injected animals 2 weeks after beginning treatment. At autopsy, the muscle underlying these ulcers was found to be heavily congested over an area 3 to 4 times the size of the ulcer. At no time did any of the ulcers become visibly infected. After about a week the ulcers healed completely, leaving a scar covering the site of injection.

All the guinea pigs reacted to 0.1 mg O. T. (Mantoux) throughout the experiment. There were no significant differences between the weight curves of the different groups of animals. Comparison of the hemoglobin 20 and 70 days after treatment was begun disclosed a 5 to 10% decrease. This decrease in hemoglobin is usually found in tuberculous animals after the disease has become widespread and progressive. No differences between the guinea pigs treated subcutaneously or orally were noted. Average concentrations of Compound 13 present in the blood are summarized in Table III. In terms of the previously reported *in vitro* activity[2] adequate concentrations of the drug were present at all times in the circulating blood, but as will be seen, these levels are completely ineffective in the treatment of experimental tuberculosis

The amount of tuberculosis present at autopsy, and the percent of the animals which survived the full 90 days of the experiment, are listed in Table IV. Inspection of this table discloses no significant differences between the different groups either in the degree of tuberculosis or in the number of animals surviving. The same results were observed on histological examination of representative sections.

*Discussion.* The description by Dubos and Davis[1] of a liquid, synthetic medium in which pathogenic mycobacteria grow rapidly has provided investigators in the field with a most useful tool. In our hands, it has provided consistent and reproducible results. We, therefore, agree with the observation[11] that, "The use of Tween 80 medium has greatly facilitated the study of the effects of antibacterial agents upon mycobacteria." It was noted above that the findings with 2-butoxy-5-aminopyridine sodium formaldehyde bisulfite are completely different from those previously reported,[2] and it was pointed out that the difference was due to important differences in technic. The results of the *in vivo* studies with this substance indicate that the drug is ineffective even in doses which produce serious toxic effects as evidenced by early death and severe local ulceration. These findings serve to emphasize the fact that the type of organism and the medium used will

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[10] Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

[11] Middlebrook, G., and Yegian, D., *Am. Rev. Tub.*, 1946, **54**, 553.

TABLE III.
2-Butoxy-5-aminopyridine Sodium Formaldehyde Bisulfite (No. 13) Blood Concentrations in Guinea Pigs.*

| Group | No. | Blood taken | Compound No. 13 mg % avg | Range mg % |
|---|---|---|---|---|
| 1A | 13 | 9 A.M. | .65 | .33-1.13 |
| 2A | 12 | 1 hr after injection | 3.2 | 1.4 -5.5 |
| 2A | 9 | 3 ” ” ” | 1.0 | .6 -3.2 |
| 2A | 11 | 5 ” ” ” | .66 | .44-1.1 |
| 2A | 9 | 8 ” ” ” | .23 | .14-3.1 |

* Group 1A received Compound No. 13 in 2% concentration in the diet, and ingested it *ad libitum.*

Group 2A animals received the Compound subcutaneously, 0.17 g per kg 3 times daily at 9:00 A.M., 5:00 P.M., and 12:00 M.

TABLE IV.
Average Amount of Tuberculosis in Guinea Pigs Treated with 2-Butoxy-5-aminopyridine Sodium Formaldehyde Bisulfite (Compound No. 13).*

| Group | No. of guinea pigs | Lungs | Liver | Spleen | Lymph glands | Summary | % survivors |
|---|---|---|---|---|---|---|---|
| 1 | 19 | 1.1 | 1.7 | 1.9 | 2.5 | 1.7 | 50 |
| 1A | 14 | 1.7 | 1.7 | 2.1 | 2.6 | 2.3 | 34 |
| 2 | 18 | 1.8 | 1.5 | 1.9 | 2.9 | 1.9 | 30 |
| 2A | 13 | 1.1 | 1.4 | 2.1 | 2.1 | 1.6 | 30 |
| 3 | 13 | 1.2 | 1.2 | 1.9 | 1.9 | 1.9 | 29 |

* Groups 1-1A received Compound No. 13 in 2% concentration in the diet.

Groups 2-2A received Compound No. 13 subcutaneously, 0.17 g/kg 3 times daily at 9 A.M., 5:00 P.M., and 12:00 P.M.

Group 3—controls.

The sub-groups (A) were bled by cardiac puncture for the blood studies listed in Table III.

profoundly influence the results. When 2-butoxy-5-aminopyridine sodium formaldehyde bisulfite was tested *in vitro* using non-virulent mycobacteria growing in Proskauer-Beck medium,[2] the drug appeared to have a high order of activity. When tested against virulent tubercle bacilli growing in Dubos' medium the activity appeared greatly lessened. Since the compound is ineffective in the treatment of tuberculosis *in vivo,* it would appear that in this instance the latter method of testing gave a better indication of its *in vivo* activity.

The finding that Mandelamine (methenamine mandelate) possesses activity against virulent mycobacteria *in vitro* is of interest because this drug is known to be effective in the treatment of urinary tract infections caused by non-acid-fast bacteria.[12] Its effect upon urinary tract tuberculosis is under investigation.

*Conclusions.* 1. The synthetic medium described by Dubos and Davis provides an efficient method of studying antimycobacterial agents. 2. Of 19 compounds tested *in vitro,* 4 showed sufficient activity to warrant further investigation. 3. 2-butoxy-5-aminopyridine sodium formaldehyde bisulfite is ineffective in the treatment of experimental tuberculosis in mice and guinea pigs.

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[12] Carroll, G., and Allen, H. N., *J. Urol.,* 1946, **55**, 674.

## 16236

# Effect of Dibenamine on Blood Pressure in Normotensive and Hypertensive Subjects.

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Experimental evidence in animals showing that the sympathetic nervous system plays an important part in the mediation of reflex elevation of blood pressure has been presented by several authors.[1,2,3] In hypertensive patients, lowering of the blood pressure has been induced temporarily by means of spinal[4] or continuous caudal[5] anesthesia, and for longer duration by extensive dorsolumbar sympathectomy.[6,7,8] These facts are considered generally to support the theory that a neurogenic factor plays a primary role in the genesis of hypertension.

The clinical evaluation of this neurogenic factor has proven to be difficult. The available tests for determining the presence and degree of the neurogenic element are not satisfactory because they are non-specific or the drugs employed act too diffusely. Indeed, amytal is a central nervous system depressor, spinal and continuous caudal anesthesia block both autonomic and somatic functions, tetraethyl ammonium hydrochloride affects both sympathetic and parasympathetic ganglia[9] and depresses the resting blood pressure of both normotensive and hypertensive subjects.[10,11] The value of the results thus obtained is questionable.

A drug having a relatively prolonged effect and capable of blocking specifically sympathetic impulses would meet the requirements for a desired test. Dibenamine, a new sympatholytic agent, appears to possess these pharmacologic properties.

In order to investigate the role of the neurogenic factor in hypertension, a study of the effect of dibenamine on the blood pressure in normotensive and hypertensive subjects was undertaken.

*Methods and Material.* Dibenamine (N, N - dibenzyl - $\beta$ - chloroethylamine - hydrochloride) is a new synthetic autonomic blocking agent. Structurally it is related to the nitrogen mustards. Functionally it is a highly specific sympatholytic and adrenolytic drug.[12,13] The dibenamine* used in this investigation was supplied in ampules as a 5% sterile solution in 50% alcohol, acidified for stability. It was administered by the intravenous route. An infusion of a solution of 0.9% sodium chloride or of 5% glucose was first started. When this was flowing freely, the desired amount of dibenamine was added to the flask containing 300 to 500 cc of the saline or glucose solution and mixed thoroughly. The single dosage used averaged 5 mg per

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[1] a. Heymans, C., and Bouckaert, J. J., *C. R. Soc. de Biol.*, 1931, **106**, 471; b. Heymans, C., *Surgery*, 1938, **4**, 487.

[2] Nowak, S. J. G., and Walker, I. J., *New England J. Med.*, 1939, **220**, 269.

[3] Grimson, K. S., Kernodle, C. E., Jr., and Hill, H. C., *J. A. M. A.*, 1944, **126**, 218.

[4] Gregory, R., and Levin, W. C., *J. Lab. and Clin. Med.*, 1945, **30**, 1037.

[5] Russek, H. I., Southworth, J. L., and Zohman, B. L., *J. A. M. A.*, 1946, **130**, 927.

[6] Craig, W. M., and Adson, A. W., *Surg. Clin. North America*, 1939, **19**, 969.

[7] Peet, M. M., and Iseberg, E. M., *J. A. M. A.*, 1946, **130**, 467.

[8] Smithwick, R. H., *Arch. Surg.*, 1944, **49**, 180.

[9] Coller, F. A., Campbell, K. N., Berry, R. E. L., Suter, M. R., Lyons, R. H., and Moe, G. K., *Ann. Surg.*, 1947, **125**, 729.

[10] Birchall, R., Taylor, R. D., Lowenstein, B. E., and Page, I. H., *Am. J. Med. Sc.*, 1947, **213**, 572.

[11] Haimovici, H., unpublished data.

* Dibenamine was supplied by the Givaudan-Delawanna, Inc., Delawanna, N. J., through the courtesy of Dr. W. Gump.

[12] Nickerson, M., and Goodman, L. S., *J. Pharm. and Exp. Therap.*, 1947, **89**, 167.

[13] Haimovici, H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 486.

kg of body weight. The total single dosage, regardless of weight, never exceeded 500 mg in our present study. The infusion was given in most cases over a total period of 60 to 75 minutes. At the completion of the dibenamine administration, the vein through which it was given was flushed with 100 to 150 cc of normal saline or glucose solution. (We found this procedure necessary for preventing local phlebothrombosis, which otherwise frequently followed dibenamine infusion in the early stage of this investigation).

The effects of this drug were studied 69 times in 38 subjects (18 normotensive and 20 hypertensive). Among the latter, 14 were suffering from essential hypertension and 6 from systolic hypertension associated with arteriosclerotic cardiovascular disease.

Arterial blood pressure was measured in the arm by the standard auscultatory method. The measurements were made in the supine, sitting and standing positions. The blood pressure (B. P.) and the pulse rate (P. R.) readings were taken at frequent intervals before, during and after the infusion. In most instances the observations were carried out over a period of 24 to 72 hours. All subjects were hospitalized patients, whose blood pressure was recorded over a period of a few days or weeks before the present tests were undertaken. In addition, the study of B. P. changes preceding and following dibenamine administration was done under standard, basal conditions. After the infusion the patients were kept in bed for about 12 to 24 hours.

*Results. A. Effect of dibenamine on the blood pressure in the supine position.*

1. Thirty tests were performed in 18 *normotensive* subjects (Table I). The average pre-injection B.P. was 124/73, the average post-injection B.P. was 122/69, with an average change of –2/–4. The range of variations in the post-injection B.P. was between +30 and –40 for the systolic and between +20 and –24 for the diastolic.

2. In *hypertensive* subjects the effect of dibenamine varied with the type of hypertension. The results obtained fall into 3 groups.

a. The first group (Table I) includes those patients with essential hypertension whose B. P. fell to normal or below normal levels. Six out of the 7 subjects in this group had an uncomplicated essential hypertension (benign phase) and one had visceral changes characteristic of the malignant phase of the disease. In the 17 tests performed in this group, the average pre-injection B. P. was 180/112, the average post-injection B. P. was 132/86, with an average change of –48/–26.

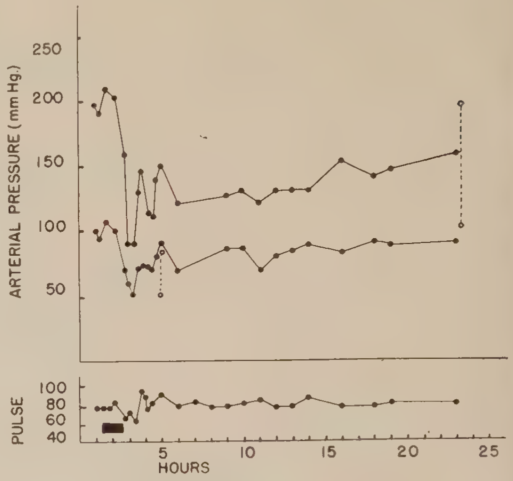

Fig. 1.

Typical effect of dibenamine (■) on the resting blood pressure and pulse rate in a case of benign essential hypertension. The vertical broken lines represent orthostatic blood pressure changes after one minute in the upright position.

The onset of the depression on the B. P. occurred occasionally during the infusion, but in most instances the fall began at or soon after the conclusion of the drug administration. The maximum fall was recorded usually within 30 to 60 minutes after the onset of the depression. However, in 2 instances a delayed response was seen, and the maximum depression of the B. P. was noted 2½ and 4½ hours respectively after the completion of the dibenamine infusion.

The return of the supine B. P. to the pre-injection level was gradual and took usually from 24 to 72 hours. Fig. 1 illustrates a typical response of a patient with moderately advanced essential hypertension.

The depressor effect of dibenamine was usually more marked after the second and subsequent injections than after the first one,

TABLE I.
Effect of Intravenous Injection of Dibenamine on the "Resting" Blood Pressure and Pulse Rate.

| Group of patients | No. of subj. | No. of inj. | Pre-injection B.P. | Pre-injection P.R. | Post-injection B.P. | Post-injection P.R. | Change in Syst. | Change in Diast. | Change in P.R. |
|---|---|---|---|---|---|---|---|---|---|
| 1. Normotensive | 18 | 30 | 124/73 ± 2.2/1.7* | 78 ± 2.0 | 122/69 ± 3.0/1.9 | 84 ± 2.8 | — 2 ± 2.6 | — 4 ± 1.5 | +6 ± 1.6 |
| 2. Essential hypertension (benign phase†) | 7 | 17 | 180/112 ±5.7/3.0 | 82 ± 2.8 | 132/86 ± 6.8/3.9 | 86 ± 3.9 | —48 ± 3.6 | —26 ± 2.5 | +4 ± 2.1 |
| 3. Essential hypertension (malignant phase) | 7 | 12 | 231/136 ± 7.6/5.6 | 100 ± 4.0 | 204/122 ± 5.2/3.3 | 102 ± 3.8 | —27 ± 4.3 | —14 ± 3.2 | +2 ± 1.1 |
| 4. Systolic hypertension (associated with A.S.C.V.D.) | 6 | 10 | 177/81 ± 3.8/3.6 | 82 ± 6.6 | 170/79 ± 5.9/2.7 | 83 ± 4.5 | — 7 ± 4.8 | — 2 ± 2.0 | +1 ± 3.5 |

* Mean ± Standard Deviation of the Mean.

† In this group, which responds to dibenamine, one patient presented visceral manifestations characteristic of the advanced phase of essential hypertension.

if an interval of only 2 to 4 days elapsed between the injections. This was noted even when the B. P. had returned to the pre-injection level during the interval, and might indicate a certain cumulative effect of the drug. Thus the average of the lowest B. P. after repeated dibenamine infusion was 107/75 as compared to 132/86, the average of the B. P. after all the injections.

b. The second group (Table I) includes 7 patients with essential hypertension whose B. P. did not change significantly after the administration of dibenamine. All the patients in this group had clinical manifestations of the malignant phase of essential hypertension (neuroretinitis, impairment of the renal function, E.C.G. changes indicative of myocardial damage, etc.). The effect of dibenamine on the B. P. in this group was either a small depression, no change or even a slight rise over the pre-injection values. When a fall in B. P. did occur, neither the systolic nor the diastolic pressures ever reached a normal level. The average pre-injection B. P. was 231/136, while the average post-injection B. P. was 204/122.

c. The third group of hypertensives includes 6 patients with systolic hypertension and arteriosclerotic cardiovascular disease (Table I). In this group the B. P. exhibited only slight variations, and the average pre-injection and post-injection values were practically the same (177/81 and 170/79).

*B. Orthostatic changes.* In most instances marked postural hypotension, accompanied by marked tachycardia (140 to 160), was seen following the intravenous administration of dibenamine, in both normotensive and hypertensive subjects. The orthostatic changes were present usually at or soon after the completion of the infusion and disappeared 12 to 24 hours thereafter. As a rule postural tachycardia outlasted by about 12 hours postural hypotension.

*C. Other effects.* The range of variations in the resting pulse rate was between +36 and –20, with an average change of +3. This applied to all groups of patients.

Measurement of skin temperatures of the head and the upper and lower extremities, under control conditions, were recorded in all cases. Release of the sympathetic tone was apparent first in the head, second in the upper extremities, and last in the lower extremities. Delayed or absent responses were noted in the lower extremities of certain patients.

Observations on these effects and others (pupillary changes, sweat function, pain, etc.) will be reported elsewhere.

*D. Side Effects.* Side reactions due to the administration of dibenamine were either local or general. Local reactions included phlebothrombosis, which has been mentioned already under methods and materials, and pain in the arm along the vein during the infusion, which was seen in 4 instances. Most general reactions were minor. Dryness of the mouth and congestion of the nose were seen in practically all cases. Drowsiness and transient nausea were common. The major side effects included vomiting in 8 instances, mental confusion and emotional lability in 3 cases, and one case of mental confusion with loss of sphincteric control and deep sleep lasting one hour.

All side effects were transient. A slow, even rate of infusion and confinement to bed of the patient for about 12 hours after the injection, *i. e.* until the orthostatic hypotension could no longer be elicited, appeared important factors in the prevention or reduction of the side effects to a minimum.[†]

*Comments.* The administration of dibenamine resulted in a significant reduction of the resting B. P. of subjects with benign or moderately advanced essential hypertension only. These results are at variance with those presented by Hecht and Anderson.[14] Orthostatic hypotension occurred in both normotensive and hypertensive subjects.

There is ample evidence to show that dibenamine is a very potent and highly specific adrenolytic and sympatholytic agent.[12,13] It probably acts directly on the neuro-effector cells, where it blocks the sympathetic impulses or the action of sympathomimetic substances (sympathin E, epinephrine). Thus the B. P. changes induced by dibenamine may be interpreted as being due to the blockade of sympathetic tone.

The lowering to normal levels of the resting blood pressure by dibenamine, in subjects with benign or moderately advanced essential hypertension only, tends to suggest that: 1. a neurogenic element, *i. e.* altered sympathetic tone is present in essential hypertension, and 2. the evaluation of this element by the administration of dibenamine may be of great help in the selection of patients when surgery is contemplated.

Because of its specific sympatholytic action, dibenamine offers a valuable tool for the investigation of the neurogenic component of a variety of vascular conditions. Further study is under way.

*Summary.* 1. Dibenamine hydrochloride, a new sympatholytic and adrenolytic drug was administered intravenously in the dosage of 5 mg/kg of body weight to normotensive and hypertensive subjects. 2. Reduction to normal or below normal levels in resting arterial blood pressure was observed only in benign or moderately advanced essential hypertension, but not in the malignant phase. Orthostatic hypotension occurred in both normotensive and hypertensive subjects. 3. The depressor effect started at the end of the infusion and lasted 24 to 72 hours. 4. All side reactions were transient, and major side reactions occurred only in a few instances. 5. The role of the neurogenic factor in essential hypertension is discussed. The usefulness of dibenamine in evaluating the neurogenic element is suggested.

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[†] Much care was exercised by keeping the patient in bed during the phase of orthostatic hypotension because of the possible deleterious effect of the latter on the coronary circulation in advanced cardiovascular disease.

[14] Hecht, H. H., and Anderson, R. B., *Am. J. Med.*, 1947, **3**, 3.

### 16237

# Early Effects of Denervation Upon Response of Muscle to Continuous Stimulation.

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The gastrocnemius muscle of the normal rat can sustain a high rate of work for several days in response to direct faradic stimulation.[1] The purpose of this study was to determine the early effects of cutting the sciatic nerve upon the ability of the gastrocnemius muscle to sustain its responsiveness. It was found that the response of the denervated muscle to a standard stimulus was decreased below that of sham-operated animals within 24 hours after the nerve was cut and continued to decrease rapidly until responsiveness was almost completely lost.

*Methods.* Male rats of the Sprague-Dawley strain were used. The control and experimental animals were closely matched into pairs according to weight which ranged from 310 to 350 g. The sciatic nerve was cut just above the level of the knee joint. In the control animals the nerve was exposed but not cut. The operations in Exp. 1 were done under barbiturate anesthesia, and the animals were subjected to the work test immediately following operation. The operations in Exp. 2 and 3 were done under ether anesthesia, since there was a delay of 24 to 48 hours in starting the work test. The animals were prepared for the work test by the subcutaneous injection of phenobarbital sodium and the intraperitoneal injection of cyclopal sodium. Anesthesia was maintained throughout the experiment by the repeated subcutaneous injection of phenobarbital sodium.

The animals were placed upon an animal board, enclosed in a temperature cabinet (28°C) and prepared for the stimulation of muscle to lift 100 g 3 times per second, according to the procedure of Ingle.[2] A silver needle placed in the biceps of the thigh served as one electrode, and a hemostat, which was clamped on the lower tibia, served as the second electrode. The muscular contractions were registered on automatic work adders. The relative amounts of work are expressed as recorded revolutions, and rates of work as number of recorded revolutions per minute (RPM). Each recorder revolution represents approximately 400 g-cm of work. All of the apparatus used was refined over that previously described[2] but the principles of method have remained the same. An electrical stimulator (Nerve Stimulator, Model B, Upjohn) was used which had adjustable amplitude, wave shape, pulse length and pulse frequency. A cathode ray oscilloscope was incorporated into the stimulator for observing and calibrating the output. A small amount of AC current was imposed on that of the DC pulse. The frequency of pulse was 3 per second, its duration was 20 milliseconds and its intensity was 12 milliamperes.

*Experiments and Results.* In Exp. 1, 12 rats having the sciatic cut and 12 rats having sham operations were subjected to direct stimulation of the muscle immediately following operation. The rates of work were recorded at the beginning of the experiment and 24, 30, 36, 48, 54, 60, and 72 hours later. The average rate of work for denervated muscle was depressed below that of the control animals at 24 hours and decreased rapidly thereafter so that only feeble responses were recorded after 48 hours. At the end of the 72-hour test period the intensity of the stimulus was increased from 12 to 20 milliamperes. The average rate of work increased from 0.5 to 13.2 RPM in the denervated muscles and from 10.2 to 15.7 RPM in the control series. The total number of recorder revolutions for the denervated series was 24,764 and for the control series 51,655.

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[1] Heron, W. T., Hales, W. M., and Ingle, D. J., *Am. J. Physiol.*, 1934, **110**, 357.

[2] Ingle, D. J., *Endocrinology*, 1944, **34**, 191.

In Exp. 2, 12 rats were denervated under ether anesthesia, and 24 hours later they were anesthetized with the barbiturates and subjected to direct stimulation of the muscle. The initial level of work was subnormal and decreased rapidly so that muscular responsiveness was almost lost by 24 hours. When the intensity of stimulation was raised to 20 milliamperes the average rate of work increased from 0.1 to 18.5 RPM.

In Exp. 3, 12 rats were denervated and 12 rats were sham-operated under ether anesthesia, and 48 hours later they were anesthetized with the barbiturates and subjected to direct stimulation of the muscle for 24 hours. The initial rates of work were normal for the sham-operated rats and subnormal for the denervated series. Muscular responsiveness decreased rapidly in the latter series so that by 24 hours muscular contractions were very weak. When the intensity of stimulation was raised to 20 milliamperes the average rate of work was increased from 1.0 to 13.7 RPM in the denervated series and from 12.8 to 21.0 RPM in the control series. The total amount of work for the denervated series was 6,638 recorder revolutions and 22,187 recorder revolutions in the control series.

The data on averages for rates of work per minute for all animals are summarized in Fig. 1.

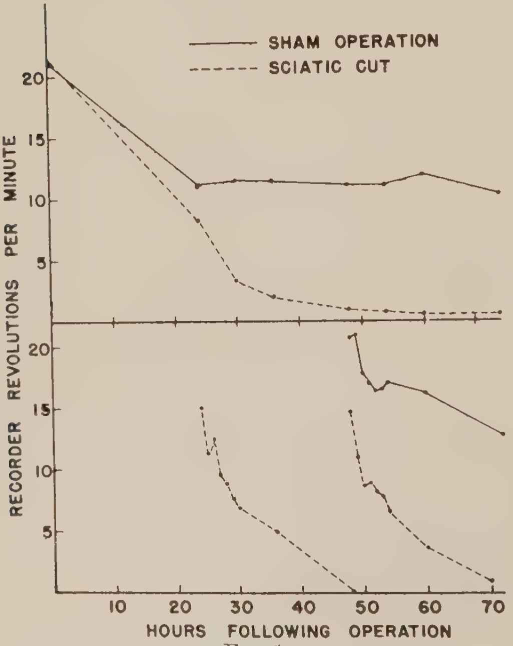

FIG. 1.
The effect of cutting the sciatic nerve upon the ability of the gastrocnemius muscle to respond to faradic stimulation.

*Discussion.* In these experiments the responsiveness of the gastrocnemius muscle was decreased below normal within 24 hours following the cutting of the sciatic nerve. The decrease occurred in those animals which were kept for 24 hours before stimulation was started as well as in the series which were stimulated continuously. The performance of work did hasten the "fatigue" of the denervated muscle, as is evident from the curves of average performance (Fig. 1) of those animals which worked continuously as compared with the performance of rats which were delayed for 24 and 48 hours following denervation.

The early effect of denervation upon the response of the muscle to direct faradic stimulation appears to be due to a rise in the threshold for adequate stimulation rather than an incapacity of the muscle to contract at all. When the intensity of the pulse was increased from 12 to 20 milliamperes at the end of the experiment the muscle contracted strongly again and sustained a high rate of work for several hours. The response was less than in the sham-operated control animals. In considering this effect it is important to recognize that our standard stimulus is optimal under ordinary experimental conditions. It will elicit an optimal amount of total work in normal animals during several days, although a temporary rise in rate of work can be induced by a more intense stimulus. A pulse of 20 milliamperes is sufficiently intense to cause tissue destruction within a period of a few hours.

The explanation of the early effect of denervation upon the ability of muscle to work is not known to us. The loss of muscular responsiveness may be due to metabolic changes caused by denervation possibly relating to the changes which result in muscular atrophy. An alternative explanation is that the dying of the nerve fibers removes an important excitatory mechanism from the muscle. Although the response of muscle to direct stimulation has commonly been described as due to the direct excitability of muscle fibers, it seems possible that the spread of the electrical pulse through the entire muscle may excite the nerve fibers which innervate it to play an important role in eliciting the response of muscle.

Separate experiments (unpublished data) have been carried out to determine the effect of curare upon the ability of the muscle to sustain responsiveness to faradic stimulation. If it were possible to completely block the myoneural junction without unfavorable effects upon respiration and circulation, the structures involved in the response to the stimulus could be further elucidated. Normal rats were given continuous subcutaneous injections of curare for periods up to 48 hours In these experiments curare did not affect work output in any dosage which did not cause respiratory failure. It was also shown that doses which failed to cause respiratory symptoms also failed to completely block the myoneural junctions in the stimulated muscle.

*Summary.* Male rats (310 to 350 g) were subjected to direct faradic stimulation of the gastrocnemius muscle following the cutting of the sciatic nerve, and their work performance was compared to that of sham-operated controls. When stimulation was started immediately following the operation the rate of work of denervated muscle was normal for several hours but was depressed below normal by 24 hours and continued to decrease rapidly, whereas the control animals sustained a high rate of work. When stimulation was delayed for 24 and 48 hours following denervation, the initial contractions were subnormal and decreased rapidly during continued stimulation. When the intensity of the pulse was increased from 12 to 20 milliamperes, at the end of each experiment there was a striking recovery in the rate of work of denervated muscle but it remained below that of the sham-operated controls.

## 16238 P

# Rodenticidal Action of 2-Chloro-4-Dimethylamino-6-Methylpyrimidine (Castrix).*

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At the close of the European phase of the war it was revealed that German scientists had developed a new rodenticide (2-chloro-4-dimethylamino-6-methyl-pyrimidine) which was known as "Castrix." Since no data on the toxicity of Castrix were available in this country and no antidote against accidental poisoning in man and domestic animals was known the present investigation was undertaken to ascertain the toxicity of Castrix to several species, to examine its efficacy as a rodenticide, and to find an effective antidote for the treatment of accidental poisoning. The results of this preliminary study demonstrated the high toxicity of Castrix and the effectiveness of pentobarbital sodium as an antidote against Castrix poisoning.

*Experimental.* In all of the species examined Castrix produced symptoms typical of central nervous system stimulants. Convulsions occurred after a latent period of 15 to 45 minutes following either oral or intraperitoneal administration. The head retracted and the fore-legs and hind-legs were tetanically extended. After the tonic phase

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* The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army, and the University of Chicago Toxicity Laboratory. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

TABLE I.
Acute Toxicity of Castrix.

| Species | Route of administration | $LD_{50} \pm$ S.E. (mg per kg) | No. of animals |
|---|---|---|---|
| Rat | Oral | $1.25 \pm 0.10$ | 30 |
| Rat | Intraperitoneal | $1.00 \pm 0.06$ | 132 |
| Mouse | ,, | $0.42 \pm 0.05$ | 82 |
| Guinea pig | ,, | $2.66 \pm 0.10$ | 65 |
| Rabbit | ,, | *ca.* 5 | 13 |
| Dog | ,, | *ca.* 0.5 | 8 |

clonic movements spread over the entire body increasing in intensity for several seconds and then subsided. Several convulsive seizures occurred intermittently terminating with death after lethal doses or complete recovery after sublethal doses.

The high toxicity of Castrix† to 5 species is shown by the data in Table I. No differences in toxicity were observed when either aqueous or aqueous ethanol solutions (10% ethanol) were employed. A comparison of the toxicity of Castrix to 20 male and 20 female rats indicated that there was no sex difference in susceptibility. No evidence of tolerance was obtained when rats were given sublethal doses for varying periods and then given a lethal dose (2 mg/kg) of Castrix. The toxicity data shown in Table I were obtained during the summer months. Subsequent tests have shown some seasonal variation in the toxicity of this rodenticide to rats since the $LD_{50}$ of Castrix to Sprague-Dawley rats increased to 2.5 mg/kg during the winter months.

To test the acceptability of Castrix when offered to rats in the food 3 groups, each containing 5 rats, were fed Wayne Dog Blox *ad libitum* and were then offered the same diet ground and mixed with varying amounts of Castrix. The acceptability and high lethality of Castrix offered in the diet are shown in Table II.

TABLE II.
Acceptability of Castrix to Rats.

| % Castrix in diet | Mortality | Time of death (hr) |
|---|---|---|
| 1.00 | 5/5 | 2 |
| 0.50 | 5/5 | <12 |
| 0.25 | 5/5 | <12 |

The symptoms of Castrix poisoning suggested that anti-convulsants might be effective antidotes. Sodium pentobarbital (Nembutal) was chosen for the present studies. Since convulsions constitute the first observable sign of poisoning, an antidote would have no practical value in man and domestic animals unless it were effective when administered during or after the onset of convulsions. Therefore, in these experiments Nembutal was never administered until the first convulsion began in about 25 minutes after administration of the rodenticide.

The data in Table III show the antidotal action of Nembutal in Castrix-poisoned rats. It is apparent from these results that at least 10 $LD_{50}$ doses of Castrix can be effectively counteracted by Nembutal. Since the animals which succumbed to 20 mg/kg of Castrix died in extreme central depression it is possible that more careful regulation of the dosage of the barbiturate might result in complete protection against 20 $LD_{50}$ doses.

Preliminary experiments with dogs have shown that Nembutal is effective in counteracting at least 15 to 20 $LD_{50}$ doses of Castrix. Since it was necessary to prolong the treatment for at least 13 hours when 10 mg/kg were given to dogs it appears that Castrix is detoxified more slowly in dogs than in rats.

*Summary and Conclusions.* A powerful convulsive agent, Castrix (2-chloro-4-dimethylamino-6-methylpyrimidine) is more toxic to rats than is either alpha-naphthylthiourea[1] or sodium fluoroacetate[2] by oral

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† We are indebted to Dr. J. R. Stevens of the Baker Chemical Co., Phillipsburg, N.J., for the Castrix used in these experiments.

[1] Richter, C. P., *J. Am. Med. Assn.*, 1945, **129**, 927.

[2] Kalmbach, E. R., *Science*, 1945, **102**, 232.

TABLE III.
Treatment of Castrix Poisoning in Rats with Sodium Pentobarbital.

| Dose of Castrix (mg/kg) | No. of rats | Pentobarbital initial dose (mg/kg) | Schedule of pentobarbital treatment Hr after Castrix 1 | 1.5-2 | 3 | 6 | % survival |
|---|---|---|---|---|---|---|---|
| 5 | 5 | 45 | 30 | 20 | | | 100 |
| 5 | 5 | 45 | 30 | | 20 | | 100 |
| 10 | 5 | 45 | 30 | 30 | | 30 | 100 |
| 20 | 5 | 30 | 30 | | 15 | | 60 |
| 20 | 5 | 30 | | 30 | 20 | 15 | 60 |

and parenteral administration. Diets containing 0.25% to 1% of Castrix are readily eaten by rats and are highly toxic. No perceptible sex difference in susceptibility and no noticeable tolerance were observed in rats but some seasonal variation in susceptibility was noted. Sodium pentobarbital is an effective antidote against at least 10 $LD_{50}$ doses of Castrix in rats and dogs even when administered after convulsions have begun.

### 16239

## Lack of a Hypoglycemic Response to Intrathecal Injection of Glucose.*

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Marinelli and Giunti have recently presented data which clearly indicated that an increase in the glucose concentration of the spinal fluid of dog and man induced a rapid and pronounced reduction in the peripheral blood sugar level.[1] Lackey failed to obtain similar results in dogs.[2] In view of the paucity of evidence concerning the mechanisms whereby the central nervous system exerts its influence on the regulation of the blood sugar level, it was considered advisable to attempt to confirm the observations of Marinelli and Giunti in man. Consequently, we studied the influence of an increase in the cerebrospinal fluid glucose concentration on the blood sugar level.

*Methods.* Subjects suffering from a variety of cerebrovascular and psychiatric disorders were studied after a preliminary fast of approximately 12 hours. Samples of blood were obtained from the antecubital vein before and at intervals after the intrathecal injection of glucose. Spinal fluid samples were obtained throughout the period of observation by means of an inlying needle inserted into the lumbar subarachnoid space. Glucose was injected into the spinal fluid in quantities of 5 to 10 ml of a 5% sterile solution in distilled water (250-500 mg) by way of either the cisternal or lumbar routes. The glucose concentrations of both blood and spinal fluid were determined by the Nelson modification[3] of the Somogyi method.

*Results.* The data obtained in this study are listed in Table I. In no instance was there noted any significant change in the blood sugar level in spite of the marked increase in the cerebrospinal fluid glucose content. It will

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* Aided in part by Research Grant No. 917 of the Research Grants Division, U. S. Public Health Service.

[1] Marinelli, L., and Giunti, V., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 23.

[2] Lackey, R. W., *Science*, 1947, **106**, 618.

[3] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

TABLE I.
Blood and Spinal Fluid Sugar Levels After Intracisternal Injection of Glucose in Man.

| Subject | Glucose injected into cisterna mg | Spinal fluid* sugar—mg % | | | | |
|---|---|---|---|---|---|---|
| | | Before | 15 min. | 30 min. | 45 min. | 60 min. |
| 1 | 250 | 62 | 70 | 63 | 64 | 61 |
| 2 | 250 | 76 | | | | |
| 3 | 400 | 57 | 1011 | 913 | 832 | 630 |
| 4 | 500 | 60 | 239 | 406 | 761 | 830 |
| 5 | 500 | 60 | 118 | 80 | 76 | 126 |
| 6 | 500 | 66 | 65 | 70 | 84 | 97 |
| 7 | 500 | 72 | 536 | 736 | 782 | 736 |
| 8 | 500 | 56 | 208 | 638 | 742 | 767 |
| | | Blood sugar—mg % | | | | |
| | | Before | 15 min. | 30 min. | 45 min | 60 min. |
| 1 | 250 | 85 | 82 | 83 | 81 | 76 |
| 2 | 250 | 88 | 96 | 94 | 97 | 104 |
| 3 | 400 | 72 | 68 | 70 | | 71 |
| 4 | 500 | 76 | 76 | 71 | 73 | 74 |
| 5 | 500 | 86 | 90 | 88 | 86 | 86 |
| 6 | 500 | 79 | 85 | 83 | 82 | 82 |
| 7 | 500 | 98 | 95 | 100 | 97 | 100 |
| 8 | 500 | 80 | 85 | 91 | 98 | 81 |

* Drawn from L4.

also be noted that the intracisternal injection of glucose was followed by a marked rise within 15 minutes in the glucose content of spinal fluid withdrawn from the lumbar region. Such a finding indicates the free diffusion of the injected glucose and may be taken as presumptive evidence that every part of the central nervous system was in contact with an increased concentration of glucose.

Since similar results have been obtained in subjects free from neurologic and psychiatric disorders, it is obvious that an increase in the glucose level of the cerebrospinal fluid does not initiate a chain of reactions resulting in a decrease of the blood sugar level. It seems fairly definite, therefore, that any mechanisms that may be involved in the regulation of carbohydrate metabolism by the central nervous system are not significantly influenced by increase in the glucose content of the spinal fluid.

*Summary and Conclusion.* Increases in the spinal fluid glucose concentration do not produce any effect on the blood sugar level.

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## 16240

# Infectivity of Sporozoites of *Plasmodium Cathemerium 3H2* Exposed *In Vitro* to Hen and Canary Bloods.*

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In this laboratory the avian malarial parasite, *Plasmodium cathemerium* 3H2, has been maintained by continuous mosquito-canary-mosquito passage since February, 1937. During all of this time there has never been a failure to infect a canary; *i. e.*, in the rare instances in which a first trial has not succeeded a second trial has always done so. But

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* This research was supported in part by a grant from the National Institute of Health.

it has not been possible to infect with this organism the great horned owl,[1] the guinea pig,[2] the barnyard hen, or man.[3] In this present communication there will be recorded the results of an attempt to determine whether the blood of the hen contains a factor inhibitory to the sporozoites of *P. cathemerium* 3H2 when they are exposed to it *in vitro*. For the purposes of the study the sporozoites were incubated separately but simultaneously for ½ to 2½ hours in hen and canary bloods, and then the blood-sporozoite mixtures were injected intramuscularly into susceptible canaries.

*Method.* Using a tuberculin syringe to which was attached a 24-gauge ¾-inch needle filled with 1% sodium heparin solution preserved with 0.5% phenol, 0.2 cc of blood was drawn from the heart of each of 8 canaries to make a total of 1.6 cc. The blood was pooled as drawn in a rubber-stoppered 5-cc vaccine bottle held in the mouth of the operator and kept in motion by a frequent bobbing of the head, both movement of the blood and fair maintenance of temperature being accomplished by this maneuver. The blood was drawn in about 10 minutes total elapsed time and the bottle was then immediately placed in an incubator at 41.5°C, being affixed in the incubator to a rotor making 1 complete revolution per minute. Immediately after bleeding the canaries, an amount of blood—7 cc—thought to be equivalent to the fraction of the total drawn from the canary was taken by heart puncture from each of 3 hens, using a 3-inch "Bishop B-19" needle containing heparin solution in amount proportionate to that employed in bleeding the canaries. At once, 0.5 cc of each of 2 of these hen blood samples and 0.6 cc of the third were placed together in a 5-cc vaccine bottle, making a total of 1.6 cc as in the case of the canary blood. The pool of hen blood was usually prepared and in the incubator rotor in 5 to 8 minutes. Then as quickly as possible the sporozoites were prepared for addition to the bloods, as follows: 100 *Culex pipiens* mosquitoes that had taken an infective blood meal 2 weeks previously were stunned by rapping the Kjeldahl flask in which they were contained a number of times against the palm of the hand, after which they were dumped into a small evaporating dish containing 1.0 cc of Locke's solution. With a fairly large pestle the mosquitoes were then ground for precisely 30 seconds; the triturate was then transferred to 3 layers of gauze in a small funnel protruding through a stopper into a calibrated centrifuge tube and washed through the gauze with sufficient Locke's solution to bring the filtrate up to the 1.0 cc mark in the tube. Then the bottles of blood were taken out of the incubator and 0.4 cc of the sporozoite-containing filtrate, withdrawn from the centrifuge tube through a specially devised long needle with a square end and a very fine bore, was added to each. The bottles, which now contained 2 cc of a sporozoite-blood mixture, were at once returned to the incubator and rotation resumed. Thereafter, at 30-minute intervals through 2½ hours, the bottles were taken from the incubator just long enough to withdraw 0.2 cc of the contents of each into separate tuberculin syringes, having 27-gauge ½-inch needles attached. At each withdrawal period 4 clean canaries were injected intramuscularly with the canary blood-sporozoite mixture and 4 with the hen blood-sporozoite mixture; each bird received 0.05 cc, an amount calculated to contain the sporozoites from 1 mosquito. The peripheral blood of the injected canaries was examined for the presence of plasmodia on the 6th to 16th days, inclusive, following the injections, and thereafter any canaries in whose blood organisms had not appeared were challenged by the bites of infected mosquitoes to determine whether or not they were still susceptible to malarial infection.

*Results.* Tables I and II comprise the findings in this study. The 4 separate trials were made a sufficient number of weeks apart to permit a full cycle of events to have transpired in the insectory so that each trial was done with a new and freshly infected lot of mosquitoes. In Table I there is ex-

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[1] Beckman, H., and Ota, R. K., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 477.

[2] Beckman, H., and Smith, J., unpublished findings.

[3] Beckman, H., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 401.

TABLE I.
Infectivity of Sporozoites of *P. cathemerium* 3H2 Exposed *in vitro* to Hen and Canary Bloods. (Data from a Single Trial.)

| Exposure period (hr) | Type of blood | Bird No. | First day of positive blood | Type of blood | Bird No. | First day of positive blood | Result of mosquito challenge |
|---|---|---|---|---|---|---|---|
| ½ | canary | 1605 | 9 | hen | 1497 | None | Infected |
| | ” | 1126 | 9 | ” | 1665 | ” | ” |
| | ” | 1843 | 8 | ” | 1983 | ” | ” |
| | ” | 1821 | 8 | ” | 1777 | ” | ” |
| 1 | ” | 1579 | 11 | ” | 822 | ” | ” |
| | ” | 1171 | 10 | ” | 817 | ” | ” |
| | ” | 1482 | 10 | ” | 1141 | ” | ” |
| | ” | 69 | 8 | ” | 1024 | ” | ” |
| 1½ | ” | 1827 | 10 | ” | 1856 | ” | ” |
| | ” | 1679 | 10 | ” | 801 | ” | ” |
| | ” | 1637 | 8 | ” | 850 | ” | ” |
| | ” | 1713 | 9 | ” | 961 | ” | ” |
| 2 | ” | 91 | 10 | ” | 840 | ” | ” |
| | ” | 1086 | 12 | ” | 1073 | ” | ” |
| | ” | 1642 | 9 | ” | 1956 | ” | ” |
| | ” | 818 | 11 | ” | 878 | ” | ” |
| 2½ | ” | 61 | 9 | ” | 1563 | ” | ” |
| | ” | 1144 | 13 | ” | 1096* | | |
| | ” | 1604 | 10 | ” | 1555 | ” | ” |
| | ” | 831 | 11 | ” | 76 | ” | ” |
| Totals: | Birds injected | | 20 | | Birds injected | 19 | All infected |
| | ” infected | | 20 | | ” infected | 0 | |

* Traumatic death on day of injection.

hibited the data from a single trial; it will be noted that organisms were found in the peripheral blood in 8 to 13 days in all of the birds injected with sporozoites in canary blood and that no organisms were found at any time in those injected with sporozoites in hen blood; it will be further observed that all of the latter group of birds did reveal organisms in the peripheral blood when they were subsequently bitten by infected mosquitoes. In Table II, comprising a summary of all the trials, it will be seen that of the 80 birds injected with sporozoites in canary blood 97.5% became infected, while of the 76 birds injected with sporozoites in hen blood only 2.6% became infected; it will also be noted that all 74 of the latter group of birds became infected when subsequently challenged by infected mosquitoes.

*Discussion.* The findings in the present study establish the fact that the blood of the hen, an animal not susceptible to infection with *P. cathemerium* 3H2, exerts an inhibiting action upon the sporozites of this organism *in vitro*. Coggeshall,[4] stating that there is considerable experimental evidence in support of the assumption that an immune serum can act upon a parasite without the participation of the cells of the host, showed that some sera from monkeys that had survived a *P. knowlesi* infection completely inhibited the infectiousness of the erythrocytic form of the parasite when it was incubated with them for ½ hour. He showed that the protective antibodies in such a serum can be removed by absorbing them by the addition of living parasites and then removing both by centrifugation, but his experimental arrangement did not lend itself to a study of the effect of these sera upon sporozoites. It seems from the work of Boyd[5] that the immune mechanism engendered by an attack of vivax malaria in the human is specifically directed toward the trophozoites

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[4] Coggeshall, L. T., *Medicine*, 1943, **22**, 87.

[5] Boyd, M. F., *J. Nat. Mal. Soc.*, 1947, **6**, 12.

TABLE II.
Infectivity of Sporozoites of *P. cathemerium* 3H2 Exposed *in vitro* to Hen and Canary Bloods.
(Summary of all trials.)

| | Canary blood | | Hen blood | | |
|---|---|---|---|---|---|
| Trial No. | Birds injected | Birds infected | Birds injected | Birds infected | Result of mosquito challenge |
| One-half Hour Exposure. | | | | | |
| 1 | 4 | 4 | 2* | 0 | 2 infected |
| 2 | 4 | 4 | 4 | 0 | 4 " |
| 3 | 4 | 4 | 4 | 0 | 4 " |
| 4 | 4 | 4 | 3* | 2 | 1 " |
| One Hour Exposure. | | | | | |
| 1 | 4 | 3 | 4 | 0 | 4 " |
| 2 | 4 | 4 | 4 | 0 | 4 " |
| 3 | 4 | 4 | 4 | 0 | 4 " |
| 4 | 4 | 4 | 4 | 0 | 4 " |
| One and One-half Hours Exposure. | | | | | |
| 1 | 4 | 4 | 4 | 0 | 4 " |
| 2 | 4 | 4 | 4 | 0 | 4 " |
| 3 | 4 | 4 | 4 | 0 | 4 " |
| 4 | 4 | 4 | 4 | 0 | 4 " |
| Two Hours Exposure. | | | | | |
| 1 | 4 | 4 | 4 | 0 | 4 " |
| 2 | 4 | 4 | 4 | 0 | 4 " |
| 3 | 4 | 3 | 4 | 0 | 4 " |
| 4 | 4 | 4 | 4 | 0 | 4 " |
| Two and One-half Hours Exposure. | | | | | |
| 1 | 4 | 4 | 4 | 0 | 4 " |
| 2 | 4 | 4 | 3* | 0 | 3 " |
| 3 | 4 | 4 | 4 | 0 | 4 " |
| 4 | 4 | 4 | 4 | 0 | 4 " |
| Totals | 80 | 78 | 76 | 2 | 74 " |

Percentage infected after exposure to canary blood 97.5
" " " " " hen " 2.6

* Numbers less than 4 reflect early deaths from injection trauma.

(*i. e.,* the erythrocyte phase) and not toward the sporozoites. Mulligan, Russell and Mohan[6] reported that the sera of many species of normal animals and man agglutinate the sporozoites of *P. gallinaceum* in low dilutions but that agglutination in high dilutions occurred with only the sera of animals or men having chronic malarious infections. It was not determined in the present study whether the effect of the hen's blood upon the sporozoites was an agglutinative one. Huff and Coulston,[7] failing to find cryptozoites in canaries inoculated with the sporozoites of *P. gallinaceum,* made the assumption that the barrier must exist in macrophages and other phagocytic cells or in antagonistic humoral substances. Since in the present study it is shown that canaries injected with sporozoites that have been exposed to hen blood not only fail to become infected but can later be infected by sporozoites that have not had such contact, it seems likely that hen's blood depresses the sporozoites sufficiently to prevent the initiation by them of the exoerythrocytic cycle.

*Summary.* Canaries were injected intramuscularly with sporozoites of *P. cathemerium* 3H2 in pooled hen or canary blood, the sporozoite-blood mixtures having been incubated previously at 41.5°C, with agitation, for periods of ½, 1, 1½, 2, and 2½ hours. Of 80 canaries injected with sporozoites in canary blood, 97.5% became infected; of 76 canaries injected with sporozoites in hen blood, 2.6% became infected. All 74 canaries which failed to become infected when injected with

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[6] Mulligan, H. W., Russell, P. F., and Mohan, B. N., *J. Mal. Inst. India,* 1940, **3**, 513.

[7] Huff, C. G., and Coulston, F., *J. Infect. Dis.,* 1946, **78**, 99.

sporozoites in hen blood did become infected when subsequently bitten by infected mosquitoes. It is felt that these findings suggest the existence of a factor in the blood of the hen that prevents the initiation by the sporozoite of the exoerythrocytic cycle in the canary.

*Subsequent note:* Further studies, completed after the above manuscript was submitted for publication, have revealed that (a) the inhibitory action of hen's blood on the sporozoites is exerted though sodium citrate is used as anticoagulant instead of heparin; and (b) it is not lessened by agitating the blood at room temperature for an hour before adding the sporozoites to it.

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## 16241 P

# Activity of Hexokinase Preparations from Rat Muscle.*

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Hexokinase activity involves the conversion of an easily hydrolyzable phosphate group to one not easily hydrolyzable (glucose-6-phosphate). Since the $P_7$† value of the reaction mixture includes both the $P_0$† and the $\Delta 7P$† but not the P in glucose-6-phosphate, any conversion of $\Delta 7P$ to glucose-6-phosphate will be reflected in a decrease in the $P_7$ values during incubation. However, the calculation of hexokinase activity in complex systems by means of such changes poses a difficult problem since transphosphorylation from ATP may fail to account for all of the phosphate changes actually occurring.

In rat muscle preparations at least two other types of transfer, in addition to transphosphorylation, are often observed. Large increases in inorganic phosphate may occur, even in the presence of fluoride, due to the dephosphorylation of ATP by the apyrases[1] present in such preparations. In addition part of the inorganic phosphate formed during incubation may be derived from sources other than the $\Delta 7P$. The action of the apyrases is accompanied by a large increase in the $P_0$ content, a decrease in the $\Delta 7P$ and no change in the $P_7$ value. The increase in inorganic phosphate from extraneous reactions is manifested by increases in both the $P_7$ and $P_0$ values. The increase in the $P_7$ value indicates that fraction of the total increase in inorganic phosphate that is attributable to the dephosphorylation of esters other than those containing easily hydrolyzable phosphate.

In order to demonstrate hexokinase activity by means of phosphorous transfers, it is essential to compare the changes occurring during incubation in the absence and presence of glucose. If the addition of glucose failed to influence phosphate changes other than those occurring as the result of the transphosphorylation catalyzed by hexokinase, calculation of the activity from a determination of the effect of glucose on the easily hydrolyzable phosphate content would be entirely feasible.[2] There is good evidence, however, that the presence of glucose may influence other reactions. Meyerhof and Geliazkowa[3] demon-

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† The following symbols are used: $P_0$—inorganic phosphate determined prior to hydrolysis; $P_7$—inorganic phosphate content determined after 7 minutes' hydrolysis at 100°C in 1 N HCl; $\Delta 7P$—that fraction of esterified phosphate converted to inorganic phosphate by 7 minutes' hydrolysis at 100°C in 1 N HCl (*i. e.*, $P_7 - P_0$).

[1] Meyerhof, O., *J. Biol. Chem.*, 1945, **157**, 105.

[2] Colowick, S. P., and Price, W. H., *J. Biol. Chem.*, 1945, **157**, 415.

[3] Meyerhof, O., and Geliazkowa, N., *Arch. Biochem.*, 1947, **12**, 405.

TABLE I.

Calculation of Hexokinase Activity in Extracts and Homogenates of Rat Muscle.

Each flask contained the following components: Side arm—0.1 ml 0.05 M or 0.1 M Na-ATP (pH 7.5), 0.15 ml 0.9 M NaF and 0.05 M $NaHCO_3$, 0.1 ml 1% glucose or water. Main compartment—0.4 ml 0.15 M $NaHCO_3$, 0.4 ml 0.05 M $MgCl_2$, 0.5 ml extract or homogenate and $H_2O$ to 2.05 ml. Incubation for 30 min. in 95% $N_2$, 5% $CO_2$ at 30°C. All values are expressed in terms of µg per ml of reaction mixture. The examples given are typical of a great number of similar results.

| Preparations | Glucose | Before incubation | | | After incubation | | | Hexokinase activity expressed as decrease in $P_7$ during incubation | | Hexokinase activity expressed as decrease in $P_7$-$P_0$ during incubation | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $P_0$ | $P_7$ | $P_7$-$P_0$ | $P_0$ | $P_7$ | $P_7$-$P_0$ | | (B)-(A) | | (B)-(A) |
| Extract | —— (A) | 104 | 231 | 127 | 127 | 235 | 108 | —4 | | 19 | |
| | Present (B) | 104 | 231 | 127 | 104 | 186 | 82 | 45 | 49 | 45 | 26 |
| Homogenate | —— (A) | 119 | 440 | 321 | 230 | 458 | 228 | —18 | | 93 | |
| | Present (B) | 119 | 440 | 321 | 217 | 426 | 209 | 14 | 32 | 112 | 19 |

strated that, in both homogenates and extracts of brain tissue, dephosphorylation of ATP occurs to a much greater degree in the absence than in the presence of glucose. We[4] reported similar findings in the case of homogenates and extracts obtained from rat muscle. In the presence of fluorides the activity of the apyrases although markedly inhibited still remains appreciable. The inclusion of glucose in the reaction mixture enables the hexokinase reaction in extracts to proceed through its successful competition with the apyrases for the participation of ATP. Obviously, since the presence of glucose results in an inhibition of apyrase activity, calculation of the hexokinase effect by a comparison of the changes in the Δ7P after incubation in the absence and presence of glucose represents the summation of hexokinase and apyrase activity. On the other hand, although apyrase activity is accompanied by a decrease in the Δ7P and an increase in the $P_0$, it fails to influence the $P_7$ values. It is therefore perfectly justifiable to calculate hexokinase activity from a comparison of the $P_7$ changes during incubation in the presence and absence of glucose provided that these two reactions are the only ones occurring which involve phosphate transfers to an appreciable degree.

As a matter of fact, in rat muscle extracts of the type described by Colowick, Cori, and Slein,[5] the sole phosphate changes observed to any degree are those due to these two reactions. In homogenates, on the other hand, extraneous phosphate formation is also observed. Since this latter type of transfer involves changes in both the $P_7$ and $P_0$ values, calculation and demonstration of hexokinase activity in homogenates forms an almost insoluble problem.

Since the method of obtaining hexokinase-containing extracts, as described by Colowick *et al.*,[5] yields preparations which fail to present such complex problems, it is obvious that this type of extract should preferably be employed for the demonstration of activity.

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[4] Broh-Kahn, R. H., and Mirsky, I. A., *Fed. Proc.*, 1947, **6**, 83.

[5] Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, 1947, **168**, 583.

In view of the considerations noted above, attempts to estimate hexokinase activity in homogenates may be of little avail in spite of the fact that such preparations may contain large amounts of the desired system. The data in Table I illustrate these points.

*Summary.* The activity of apyrase systems and extraneous phosphate formation interfere with the determination of hexokinase activity in rat muscle homogenates. Hexokinase activity in rat muscle extracts is best calculated from changes in the $P_7$ values during incubation, a procedure which avoids the complication of the inhibition of apyrases by hexokinase activity.

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## 16242

## Anemia in Rats Infested with *Bartonella muris* and Injected with Pteroylglutamic Acid.[*]

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An active infestation of *Bartonella muris* in rats leads to an acute anemia, hemoglobinuria, emaciation, and usually death.[1-4] In our experience the number that die may be reduced somewhat by injecting the infested blood into the rat several days or weeks before the spleen is removed rather than giving it immediately after splenectomy. Death rate has varied considerably in our rats, being around 50% a few years ago[4,5] and about 25% in the present study. Several efforts have been made to discover a satisfactory treatment for this disease. Some substances such as arsenicals,[6] extracts of spleen, copper, and iron,[7] have been found to be beneficial. Negative results were obtained in the treatment of the infestation with sulfanilamide.[4]

Since pteroylglutamic acid (PGA, folic acid) was found to be helpful in anemias of different types[8,9] it was decided to try it in rats made anemic by *Bartonella muris* infestation.

The rats used in these experiments were nearly full grown albino males fed on Purina laboratory chow (containing 2.4 $\mu$g of PGA per g).[†] They ate approximately 15 g per day at the start and much less during the severe anemia. They were injected intraperitoneally, with saline containing blood taken from rats suffering from acute infestation of *Bartonella muris*. Several days to weeks later they were prepared for operation, etherized and the spleen removed. Hemoglobin estimations and red cell counts were done every 3 to 6 days for several weeks. The hemoglobin was determined by the Sahli method. These readings were checked with the photoelectric acid hematin method for hemoglobin and found to be consistently only slightly higher. The Sahli method was there-

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[*] Research Paper No. 876, Journal Series, University of Arkansas.

[1] Ford, W. W., and Eliot, C. P., *J. Exp. Med.*, 1928, **48**, 475.

[2] McCluskie, J. A. W., and Niven, Janet, *J. Path. and Bact.*, 1934, **39**, 185.

[3] Weinman, D., *J. Infect. Dis.*, 1938, **63**, 1.

[4] Emery, F. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 56.

[5] Emery, F. E., Bugelski, T. S., and Schwabe, E. L., *Endocrinology*, 1940, **26**, 167.

[6] Mayer, M., Borchardt, W., and Kikuth, W., *Klin. Wschr.*, 1926, **5**, 559.

[7] Perla, D., and Marmorston-Gottesman, J., *J. Exp. Med.*, 1932, **56**, 777, 783.

[8] Higgins, G. M., *Proc. Staff Meet. Mayo Clin.*, 1944, **19**, 329.

[9] Kornberg, A., Tabor, H., and Sebrell, W. H., *Am. J. Physiol.*, 1944, **142**, 604.

[†] We are indebted to Mrs. Edith S. Sims for these determinations and for the PGA solutions used.

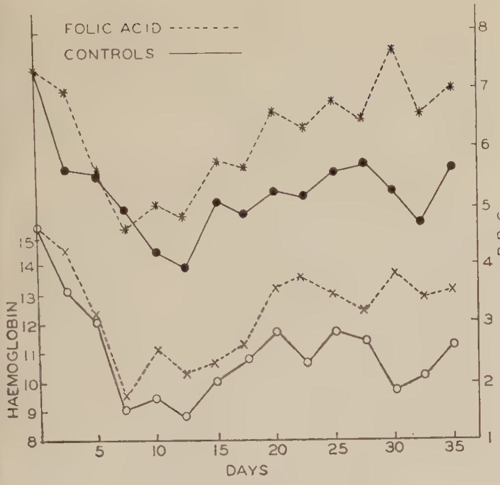

Fig. 1.

Two lower curves hemoglobin in grams of splenectomized rats infested with *Bartonella muris*. Mean values for twenty animals included in each curve. Two upper curves red blood cells in millions.

fore considered to be very satisfactory for these experiments.

Some of the rats in each group were given daily intramuscular injections of 40 μg of pteroylglutamic acid. The means (for 20 animals in each group) at intervals of 2½ days, arranged as curves of hemoglobin in grams and red cell counts in millions, are shown in Fig. 1. It will be noted that the spread between the treated and control groups is narrow for about 18 days and widens considerably thereafter. This is taken to indicate that pteroylglutamic acid aids new cell formation and in this way keeps the blood count up above the untreated rats during the progressive anemia of the Bartonella infestation; after the anemia has reached its lowest level, and blood regeneration exceeds red cell breakdown, then the injected animals have considerable more capacity for new red cell formation than the controls as the curves clearly show. This gives rise to analysis of the curve in two parts. The mean for all the hemoglobin determinations during the first 18 days in the injected rats has been compared with the mean hemoglobin content of the untreated rats and found to give a mean difference which is very significant, being more than 5 times the standard error of the difference. A similar comparison from the nineteenth to thirty-fifth day showed the mean difference in hemoglobin at this end of the curve to be over 10 times the standard error of the difference. Likewise the red cell count is also significantly higher in the rats treated with pteroylglutamic acid and the results of the analysis for this data are similar to those described for hemoglobin.

From these data it is obvious that pteroylglutamic acid is beneficial in holding the hemoglobin and numbers of red cells in the treated rats above those of the controls. From Fig. 1 it may be seen that the curves for the injected rats are above the controls as early as 2½ days after splenectomy and after injections of pteroylglutamic acid were started. The data are not numerous enough to establish a significant difference between the curves at this point. If the curves are different as early as 2½ days it would be interesting information on the function of pteroylglutamic acid and may suggest a role aside from red cell formation. On the other hand the formation of new cells in numbers sufficient to be detected in the general circulation might occur in a few days in these anemic rats. Since there was some PGA in the food these results would probably be all the more striking in rats on a PGA-free diet.

*Summary.* Hemoglobin and red cell estimations have been done on the blood of rats made anemic by splenectomy and *Bartonella muris* infestation. Animals injected with pteroylglutamic acid show hemoglobin and red cells at higher levels than those of the untreated controls. Curves made from this data show the treated animals much less anemic than the controls for a period of 35 days or longer.

### 16243

## Do Chromosomes Manifest Osmotic Volume Change?

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In an earlier study[1] it was shown that the nucleus of the immature starfish egg in the germinal vesicle stage undergoes a kinetics of swelling and shrinking which is formally similar to that of the egg as a whole. Observations made also on equilibrium volumes indicated that the germinal vesicle contains a smaller percentage of osmotically inactive material than does the cytoplasm in this cell. The advantage of studying the nucleus in the germinal vesicle stage is that its size is large enough to realize sufficiently accurate diameter measurements under high dry magnification. When the egg matures, the nucleus becomes very much smaller, and direct measurements are rendered considerably more difficult by this method.

In the spermatozoon, the chromosomes are compressed into the smallest possible volume in an animal cell, nearly devoid of cytoplasm, hence an examination of the osmotic properties of the sperm affords the nearest approach to an intracellular study of the osmotic properties of the chromosomes themselves, in the intact living cell. Since spermatozoa are not spherical, and moreover are so small, it is necessary to use some method other than direct microscopic measurement, such as centrifuging, for estimation of cell volume. Apparently the first to study this quantitatively (though not with reference to the question of chromosome swelling) was Hamburger[2] who obtained an average value of 72.8% for the "Gerüstvolum" (literally, "framework volume"; operationally, the equivalent of "osmotically inactive fraction"[3]) of frog spermatozoa. In arriving at this value, he used low centrifugal forces (1800 or 3000 r.p.m., no radius stated) and placed the sperm in solutions of pure NaCl, a procedure known to be toxic to a variety of cells.[4] It was therefore decided to investigate the matter in a species where sperm are readily available in abundance, using a balanced ionic environment and a centrifugal force adequate to pack the cells quantitatively, and to test the applicability of the Boyle-van't Hoff law.

According to Harvey and Anderson[5] the Arbacia sperm has a pointed head with a flattened base (2 μ wide) adjacent to a short slightly narrower middle piece which seems to contain a pair of spherical bodies. The length of the head and middle piece is approximately 4 μ. The length of the tail is about 45 μ. Direct observations of swelling to about twice their original size were made of sperm placed in distilled water. Electron microscope observations revealed the tail to be composed of about 10 strands of uniform thickness, each about 50 mμ in diameter. Owing to the thickness of the heads, it was not possible to make out any discrete internal structure.

*Procedure.* Sperm were obtained from ripe testes of the sea urchin, *Arbacia punctulata*, by excision from the animal, and allowing the spermatic fluid to exude as a viscous creamy suspension into a stender dish. The material was pipetted into a test tube and centrifuged lightly in order to throw down any gross tissue particles. This still left the bulk of the sperm in suspension, occupying from 23.5 to 27.8% of the total volume. Hypotonic sea water solutions, varying in concentration from 1% to 80%, were made up fresh each day. Owing to the fact that the cell volume is only a fraction of the spermatic fluid, mixture with the hypotonic sea waters yielded a range of resultant effective osmotic concentrations of 42.4 to 88.5% sea water.

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[1] Beck, L. V., and Shapiro, H., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 170.

[2] Hamburger, H. J., *Osmotischer Druck und Ionenlehre*, 1904, Wiesbaden, J. F. Bergmann, Vol. 3, p. 4, Die Volumanderungen der Spermatozoen unter den Einfluss hypertonischer und hypisotonischer Salzlosung.

[3] Lucké, B., and McCutcheon, M., *Physiol. Rev.*, 1932, **12**, 68.

[4] Loeb, J., *Pfluger's Arch.*, 1903, **97**, 394.

[5] Harvey, E. B., and Anderson, T. F., *Biol. Bull.*, 1943, **85**, 151.

The spermatic fluid was pipetted in 0.1 ml aliquots into small shell vials, an equal quantity of hypotonic sea water was added to each vial, and the contents mixed thoroughly. The shell vial was covered with a cover slip, and from 50 minutes to 2 hours allowed for the attainment of osmotic equilibrium at room temperature (about 25°C). Specially drawn capillary tubing was used, of uniform bore (approx. 0.39 mm, checked by microscope measurements of diameters at both ends) and outside diameter, 0.56 mm. The tubing was cut up into lengths of about 43 mm, and from 30 to 35 mm of diluted sperm suspension drawn up by capillarity, following which the opposite end was sealed off in a microflame. The perfection of each seal was checked by microscopic examination. Centrifuging (15 to 17 minutes duration) was carried out in an air turbine, at approximately 20,000 times gravity, in a steel rotor made available through the courtesy of Dr. A. K. Parpart. Column lengths were measured under a binocular dissecting microscope with the aid of a metric steel rule. At this centrifugal force erythrocytes are completely packed; for Arbacia eggs it has been estimated that a minimal centrifugal force of 4330 × gravity is required to pack the cells completely.[6] The exact force at which complete packing is attained will be related to the physical properties of the cell in question, and to the medium.[7]

*Condition of the Sperm.* The physiological state of the sperm cells was studied after swelling by microscopic observation of their motility and of fertilizing power. In all the dilutions employed, numerous highly active sperm were observed. As the packed sperm were allowed to rest in the capillary tube after centrifuging, the cell column length increased slightly with time, apparently due to sperm at the free end of the column migrating into the supernatant fluid. When swollen sperm were returned to a suspension of eggs in normal sea water, high percentages of membrane formation and cleavage ensued, demonstrating that the sperm had not been irreversibly affected by the swelling process.

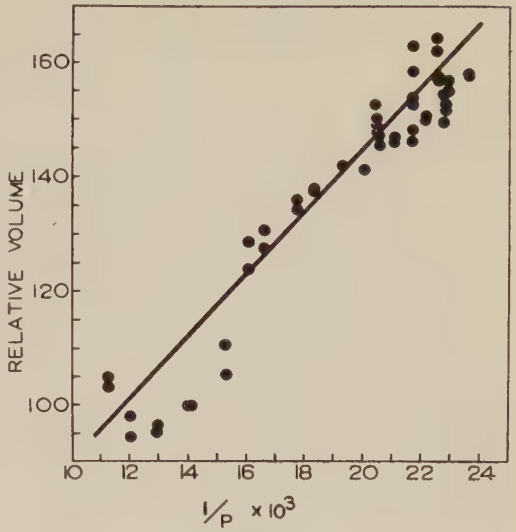

FIG. 1.

Showing the applicability of the Boyle-van't Hoff law to equilibrium volumes of sea urchin sperm swollen in various dilutions of sea water.

*Results.* Fifty-six determinations were carried out, the results of which are summarized in Fig. 1, which is a plot of relative sperm volume against the reciprocal of the osmotic pressure of the medium. A rectilinear relation indicates the applicability of the Boyle-van't Hoff law,

$$P\ (V - b) = k,$$

where P, V, b, and k represent respectively osmotic pressure, cell volume, osmotically inactive fraction, and a constant. Extrapolation of the curve to the zero ordinate (not shown) yields an average value for "b," the "osmotically inactive fraction," of 33%, a value in the same region as that observed for other cells (*e. g.*, in unfertilized Chaetopterus eggs, "b" is 38.8%.[8]) The best average curve, fitted visually, has been drawn through the data. Application of the Criterion of Least Squares shows that the line so drawn lies close to one computed by least squares. Although the plotted experimental points in Fig. 1 show a fair amount of scatter, it is essential to note that if lines with the greatest and least slope reasonably possible be drawn that "b" will have a maximum possible value of approx. 48%, and a minimum value of approx. 18%.

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[6] Shapiro, H., *Biol. Bull.*, 1935, **68**, 363.

[7] *Cf.*, for example, Johnson, F. H., and Harvey, E. N., *J. Cell. and Comp. Physiol.*, 1937, **9**, 363.

[8] Shapiro, H., *J. Cell. and Comp. Physiol.*, 1941, **18**, 143.

*Discussion.* Since the chromosomes appear to constitute the bulk of the content of the spermatozoon, it appears from this result that they must partake in the osmotic swelling of the cell. If the chromosomes did not swell, the value obtained for "b" would be very high, of the order of 90% or more, judging from the standard cytological pictures of sperm heads. In so reacting to hypotonic solutions, the chromosomes display an osmotic pattern shared by other formed elements within the nucleus and the cytoplasm, viz., the nucleolus, and the diverse types of granules which undergo osmotic volume changes. The value of 72% obtained by Hamburger for frog sperm may be due to a species difference or to the differing circumstances, alluded to in the introduction, in which the experimental determinations were carried out.

Churney[9] studied the swelling of the germinal vesicle in immature eggs of *Arbacia punctulata* and *Nereis limbata.* Unfortunately, no calculations of osmotically inactive fraction were given. He concluded that "the experiments failed to reveal any appreciable amount of osmotically inactive material in either of the nuclei." An interesting observation was also briefly reported in a footnote to the effect that "the giant chromosomes of the salivary gland cells of *Sciara coprophila* and of *Chironomus sp.* swell and shrink reversibly in anisotonic solutions." Quantitative data were not furnished.

*Summary.* A. The applicability of the Boyle-van't Hoff law to the swelling of spermatozoa of the sea urchin, *Arbacia punctulata,* in hypotonic solutions of effective concentrations varying from 42.4 to 88.5% sea water was investigated. Cell volumes were determined by centrifuging in small capillaries in an air turbine at 20,000 × gravity.

B. In the range of dilutions employed, the Boyle-van't Hoff law appeared to be followed. The "osmotically inactive fraction" ("b") averaged 33%. Thus the sperm head, which is chiefly chromosomal material, has a nonswellable volume which does not differ greatly from that of certain other cells. The results indicate that the chromosomes take part in the osmotic swelling of the cell.

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[9] Churney, L., *Biol. Bull.*, 1942, **82**, 52.

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## 16244

# An Investigation of Transmethylation from N¹-Methylnicotinamide

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With the discovery that nicotinamide is methylated in the animal body to give N¹-methylnicotinamide[1] it became of interest to know whether this product is a methyl donor in the biological synthesis of N- and S-methyl compounds. One approach to this problem has been the study of the lipotropic activity of N¹-methylnicotinamide when added to a methyl-deficient diet. Najjar and coworkers[2,3] have presented data to show that N¹-methylnicotinamide has some lipotropic action in the rat, whereas Handler and Dubin[4] found in a similar experiment that it had no activity in preventing the development of fatty livers.

We wish to report here an experiment of

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* Present address: School of Biological Sciences, The University of Tennessee, Memphis, Tennessee.

[1] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1943, **150**, 395.

[2] Najjar, V. A., and Deal, C. C., *J. Biol. Chem.*, 1946, **162**, 741.

[3] Najjar, V. A., and Ratcliffe, I. M., *Bull. Johns Hopkins Hosp.*, 1947, **80**, 142.

[4] Handler, P., and Dubin, I. N., *J. Nutrition*, 1946, **31**, 141.

TABLE I.
Feeding Experiment with N¹-deuteriomethylnicotinamide (86.0 Atom % D in the Methyl Group).

| Rat No., Sex | Change in body wt g | Total amt of test compound ingested mg | Deuterium content of isolated compounds: Choline chloroplatinate atom % | Deuterium content of isolated compounds: Creatinine potassium picrate atom % |
|---|---|---|---|---|
| 13 ♂ | 76-86 | 720 | 0.00 ± .09 | 0.00 ± .07 |
| 14 ♂ | 79-94 | 840 | 0.06 ± .13 | 0.00 ± .06 |

direct nature testing whether N¹-methylnicotinamide can function as a methyl donor in the body by labeling the methyl group of this compound with deuterium. N¹-deuteriomethylnicotinamide chloride was synthesized and fed to 2 growing rats for 6 days. At the end of this time the rats were killed and the body choline and creatine isolated. Analysis of the isolated compounds showed that no deuterium was present indicating that no transmethylation from N¹-methylnicotinamide to choline or creatine had occurred.

*Experimental. Synthesis of N¹-deuteriomethylnicotinamide chloride* ($CD_3N(Cl)$-$C_5H_4CONH_2$). This compound was prepared from deuteriomethyl iodide[5] and nicotinamide by the method of Karrer *et al.*[6] Two preparations were made, and weighed amounts of the two were mixed for the feeding experiment. For the determination of the deuterium content, the compound was burned, the water collected, purified, and the $D_2O$:$H_2O$ ratio therein was measured by the falling drop method.[7] From the results obtained it was calculated that the compound contained 86.0 atom % deuterium in the methyl group.

*Analysis.* N¹-deuteriomethylnicotinamide chloride.

Preparation 1. Calculated,† C 48.0; found, C. 47.9.
Preparation 2. Calculated, C 48.0; found, C 47.7.

*Feeding Experiment.* Two young littermate male rats were used. During a control period of 3 days they were fed *ad libitum* a diet of the following percentage composition: casein 18, sucrose 58, hydrogenated vegetable oil 19, corn oil 1, Osborne and Mendel salt mixture 4. In addition 100 g of the diet contained 20 mg choline chloride, 2 mg each of thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, and nicotinic acid, 4 mg calcium-*d*-pantothenate, 4000 U.S.P. units vitamin A, 400 U.S.P. units vitamin D, and 1 mg α-tocopherol acetate.

After the 3-day control period, 2% N¹-deuteriomethylnicotinamide chloride was put in the diet replacing an equivalent amount of sucrose. The 2 rats were pair fed. On the 6th day of feeding the test compound, rat 13 failed to eat. It was therefore killed, and autopsy showed that the kidneys were hemorrhagic. The kidneys of rat 14, which was killed at the same time, appeared to be normal. The changes in body weight and the amounts of N¹-deuteriomethylnicotinamide chloride ingested are given in Table I.

Creatine was isolated from the carcass of each rat as creatinine potassium picrate,[5] the purity of which was determined colorimetrically by the Jaffé reaction. Choline was isolated as choline chloroplatinate.[5]

*Analysis.* Choline chloroplatinate.

Rat 13—Calculated, Pt. 31.7; found, Pt. 31.7.
Rat 14—Calculated, Pt. 31.7; found, Pt. 31.5.

The isolated compounds were analyzed for deuterium by the method mentioned above, and the values obtained are given in Table I.

*Discussion.* The isolated choline and creatine contained no deuterium. Thus, under the conditions of this experiment, there was no detectable transmethylation from N¹-methylnicotinamide to choline or creatine.

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[5] du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, 1941, **140**, 625.

[6] Karrer, P., Schwarzenbach, G., Benz, F., and Solmssen, U., *Helv. Chim. Acta*, 1936, **19**, 811.

[7] Cohn, M., in Wilson, D. W., *Preparation and Measurement of Isotopic Tracers*, J. W. Edwards, Ann Arbor, 1946, p. 51.

† Calculated values are based on an increased molecular weight due to the deuterium in the molecule.

From the work of Handler and Dann,[8] and Perlzweig, Bernheim, and Bernheim,[9] as well as several more recent studies, evidence has accumulated to indicate that nicotinamide is methylated in the body by a transfer of methyl groups from a methyl donor. It would appear from the results of the present experiment as well as the experiment of Handler and Dubin[4] that this transfer must be an irreversible process. In this respect N$^1$-methylnicotinamide would be similar to creatine.[10]

It has been demonstrated in several instances that a compound may be inactive as a methyl donor and yet active as a lipotropic agent or in the prevention of hemorrhagic kidneys.[11] With regard to the latter, we found N$^1$-methylnicotinamide to be inactive. In preliminary experiments which we had carried out, we had put some young rats on a purified diet containing 2% N$^1$-methylnicotinamide with no added choline, and these rats died in about a week from hemorrhagic kidneys. With regard to lipotropic action, Handler and Dubin[4] found that N$^1$-methylnicotinamide did not prevent the development of fatty livers. On the other hand, Najjar and coworkers[2,3] observed some lipotropic action from the compound. They pointed out[3] that the slight lipotropic action which they observed might be ascribed to one of a number of factors, for example, a sparing action of the N$^1$-methylnicotinamide on the methionine and choline in the diet which they used.

It will be noted that a small amount of choline was present in the diet during the period of feeding N$^1$-deuteriomethylnicotinamide in the present experiment. The choline was included in an attempt to forestall termination of the experiment by loss of the animals from severe hemorrhagic kidney involvement. If transmethylation from N$^1$-methylnicotinamide to choline could take place one would expect the deuteriomethyl group to appear in the isolated choline even in the presence of some choline in the diet. This assumption is based on the fact that in several experiments carried out in this laboratory, transmethylation from deuteriomethionine to choline has been found to occur even when choline was present in the diet.[12]

It is of interest that trigonelline, N-methylnicotinic acid betaine, has likewise been shown to be inactive as a methyl donor.[8,11]

*Summary.* N$^1$-methylnicotinamide labeled in the methyl group with deuterium was fed to two rats for 6 days. Analysis of the body choline and creatine of these rats for deuterium showed that there was no detectable transmethylation from N$^1$-methylnicotinamide to choline or creatine during this period.

The authors wish to express their appreciation to Miss Josephine E. Tietzman for the analyses reported in this paper. The authors also wish to acknowledge a grant-in-aid from the Lederle Laboratories Division, American Cyanamid Company, which has contributed to the support of this investigation.

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[8] Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **146**, 357.

[9] Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, 1943, **150**, 401.

[10] Simmonds, S., and du Vigneaud, V., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 293.

[11] Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, 1942, **143**, 373.

[12] Schenck, J. R., Simmons, S., Cohn, M., Stevens, C. M., and du Vigneaud, V., *J. Biol. Chem.*, 1943, **149**, 355.

### 16245

## Skeletal Changes in Growing Vitamin B Complex Depleted Rats and the Course of Repair.

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In continuation of our investigations on the response of the skeletal tissues to vitamin deficiencies, we have now analyzed the growth and development of the long bones in vitamin B complex depleted young rats. The course of repair after refeeding has also been studied.

*Material and Methods.* Fifty-five rats of the Wisconsin strain (24 males and 31 females), 19 to 22 days old, were divided into 3 groups, littermates and animals of the same sex being equally distributed among these groups.

*Series I.* 12 rats were kept on water and a stock diet composed as follows:

| | g % |
|---|---|
| Yellow corn meal | 51.55 |
| Powdered whole milk | 9.15 |
| Powdered skim milk | 9.15 |
| Linseed meal | 3.86 |
| Wheat germ | 1.44 |
| Crude casein | 9.63 |
| Alfalfa leaf meal | 9.63 |
| Dried brewer's yeast | 0.96 |
| Sodium chloride | 0.39 |
| Calcium carbonate | 0.39 |
| Cod liver oil | 0.96 |
| Crisco | 2.89 |
| | 100.00 |

These rats were killed after 18, 24, 30, or 40 days.

*Series II.* 21 animals received water and the following vitamin B-free diet:

| | g % |
|---|---|
| Casein (Vit.-free) | 18 |
| Starch (Anheuser-Busch (Vit.-free) | 62 |
| Merck salt mixture No. 1 | 4 |
| Cod liver oil (Mead Johnson) | 2 |
| Crisco | 14 |
| | 100 |

The duration of the experiments was the same as in Series I, unless the poor condition of the animals necessitated an earlier sacrifice.

*Series III.* 22 rats received water and the deficient diet, but they were subsequently refed the stock diet for 5, 10, 21 or 30 days. As a rule, refeeding was begun after 3 weeks. The rats were weighed once a week. At the end of the experiment, the animals together with their controls were killed with chloroform. The upper end of the tibia and the lower end of the femur were removed as a whole, fixed in 10% formalin, decalcified in a formic acid-citrate mixture,[1] and embedded in paraffin. Slides, stained with hematoxylin and eosin, were prepared for histological studies. The width of the epiphyseal disc and of the cortex of the tibia was measured with an objectmicrometer, and average values were established. The number of trabeculae in the metaphysis of the tibia present in a standard area measuring 350 microns in diameter was counted.

*Gross Observations.* At the beginning of the observations, the mean weight of the males was 42 ± 1 g, that of the females 40 ± 6 g. During 18 days, the deficient animals, both male and female, gained 12 to 14 g, whereas the normal control males had gained 50 g and the females 68 g. Subsequently, the weights of the deficient animals dropped sharply. After 24 days, the loss was 7 ± ½ g in males and 4 ± 3 g in females, and after 30 days, 11 ± 1 g in males and 9 ± 2 g in females. The deficient state was tolerated for 34 days by 1 male that lost 18 g. Females were more resistant, 4 animals surviving for more than 30 days, and 1 for 40 days. These females lost 16 ± 4 g. After refeeding the stock diet for 5 days, the weight of the males had increased 46 ± 7 g, that of the females 40 ± 3 g, whereas the normal controls had gained 15 g in the corresponding period of time. After 10 days, the weights of the refed animals still remained 25 to 30% below those of the controls. After 21 days, the weights of the refed rats began to equal those of the normal ones, and after 30 days, the weights of 2 of the refed animals surpassed those of the controls.

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[1] Morse, A. J., *J. Dent. Res.*, 1945, **24**, 143.

*Histologic Findings. In Males.* After 18 days, the changes noted in the deficient animals were slight. The epiphyseal disc measured 320 microns (normal 355 microns). The cartilage cells were smaller and the cartilage cell rows shorter than usual. The latter were separated from each other by thin layers of chondromucoid ground substance. The single row was composed of 9 or 10 columnar and 3 or 4 hypertrophic cartilage cells (normal 12 columnar and 4 or 5 hypertrophic cells). There was mitotic proliferation of the columnar cells. The metaphysis was properly vascularized, and replacement of cartilage by bone was in progress. Whereas ordinarily the metaphyseal standard area contained 20 long osseous spicules, in the deficient animals only 16 short and thin trabeculae covered by small osteoblasts were counted. The articular cartilage cells were small. The cortex was 225 microns thick (normal 255 microns). The endosteum contained a continuous layer of oblong osteoblasts. The vascular canals were enlarged; the periosteum was dense; the bone marrow was hemopoietic.

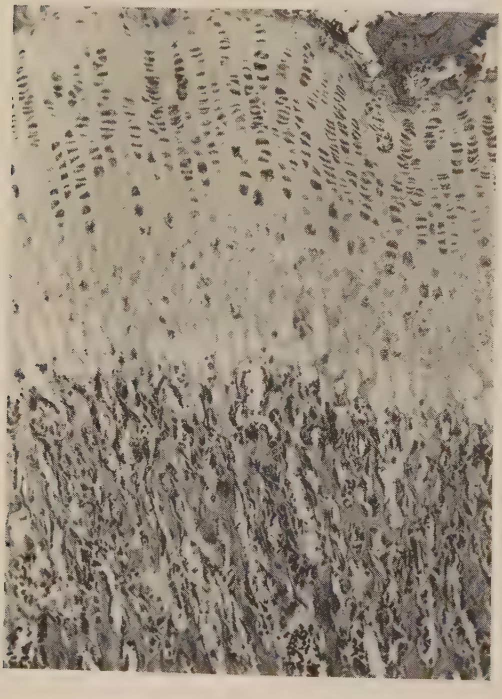

FIG. 1.
Section through the epiphyseal disc at the upper end of the tibia of a male rat, 44 days old, receiving the stock diet for 25 days. Magnification 104 ×.

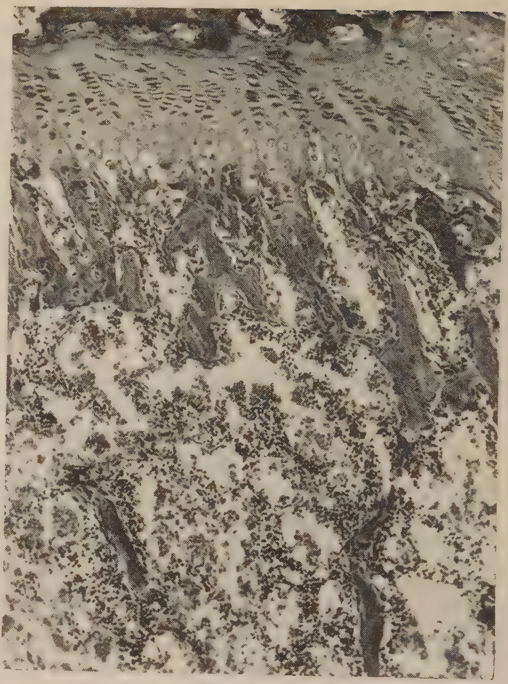

FIG. 2.
Section through the epiphyseal disc at the upper end of the tibia of a male rat, 43 days old, kept on the deficient diet for 21 days. Magnification 104 ×. Compare with Fig. 1. The epiphyseal disc is narrowed and bone formation is markedly decreased.

After 24 days, the epiphyseal discs of the deficient animals had narrowed down to 135 microns (Fig. 1, 2). The shortened cell rows were composed of 6 to 8 small columnar and 2 or 3 small sclerosed "hypertrophic" cartilage cells. Mitoses were not seen. The cartilaginous matrix was abundant and hyalinized. The vascularization of the metaphysis was diminished, and only 5 trabeculae were found in the standard area. The spicules were very short, and frequently only fragments of bone were seen. The cartilage cells of the pressure and hypertrophic zones of the joint were less numerous and smaller than ordinarily. The cortex measured 205 microns. The endosteum consisted of a discontinuous layer of small osteoblasts or spindle cells with elongated cytoplasm. The vascular canals were filled with edematous connective tissue. The periosteum was loosened. In the bone marrow, foci of serous atrophy were noted. Subsequently, the changes were still more pronounced.

After 30 days, growth of cartilage and bone was at a standstill. The single cartilage cell row contained 5 or 6 columnar and 2 small hypertrophic cells. Much hyaline matrix appeared between the cartilage cell rows, and a layer of preosseous material was seen in the distal third of the atrophic epiphyseal disc. Metaphyseal trabeculae were absent. Instead, a thin transverse bony lamella delimited the epiphyseal cartilage from the marrow cavity. The cartilaginous covering of the joint was markedly atrophic. The cortex of the shaft measured 135 microns. At its inner surface, a discontinuous layer of small spindle cells had taken the place of osteoblasts. The Haversian canals were enlarged and contained engorged vessels and edematous connective tissue. The periosteum was poor in cells. The bone marrow showed an advanced state of serous atrophy.

After 34 days, the epiphyseal disc was only 65 microns wide, and the single cartilage cell row was composed of 4 inactive columnar cells and 1 cell of hypertrophic type. The conditions were otherwise comparable to those seen after 30 days.

*In Females.* The changes were of the same kind as in the males, but their progress was slower. Thus after 18 days on the deficient diet, the epiphyseal disc was 330 microns wide (normal 340 microns), and the single cartilage cell row contained 10 or 11 columnar and 4 hypertrophic cells. The decrease in the size of the cartilage cells, however, was conspicuous. There were 18 trabeculae in the metaphyseal standard area (normal 20). The cortex was of usual diameter (245 microns). The endosteum and periosteum were unchanged.

After 24 days, the epiphyseal disc was 165 microns high. The cartilage cell rows were short and composed of 7 to 9 columnar and 4 hypertrophic cells. Both cell types were considerably smaller than usual. Hyalinization of the cartilaginous matrix was less accentuated than in the male. In the metaphysis, 8 short trabeculae covered by small osteoblasts were counted. The cortex was 225 microns thick. The changes in the endosteum and periosteum were now comparable to those seen in the male. In the bone marrow, a few foci of serous atrophy were noticeable.

After 30 days, conditions in the female had approached those in the male. After 40 days, the epiphyseal disc had narrowed down to 70 microns, the single cartilage cell row containing 3 atrophic columnar cells and 1 hyalinized "hypertrophic" cell. Trabeculae were lacking. The articular cartilage cells were markedly atrophic. The cortex of the shaft was 145 microns thick. The bone marrow had undergone complete serous atrophy.

*Effects of Refeeding.* After 5 days, the growth of the epiphyseal and articular cartilages as well as endochondral and periosteal

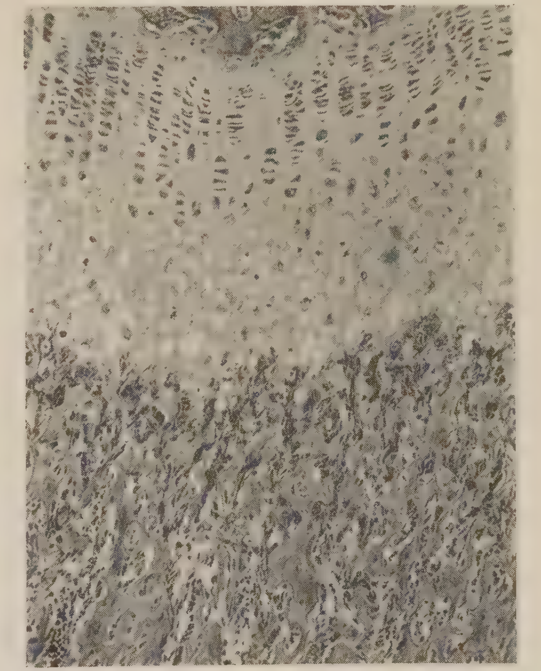

FIG. 3.

Section through the epiphyseal disc at the upper end of the tibia of a male rat, 45 days old, kept on the deficient diet for 21 days, and refed the stock diet for 5 days. Magnification 104 ×. Compare with Fig. 2. Growth of the epiphyseal cartilage and endochondral ossification are resumed.

ossification was resumed with remarkable intensity. Mitotic proliferation of the columnar cartilage cells was accentuated, and their conversion into hypertrophic cells was intensified. In the male, the epiphyseal disc was 355 microns high (Fig. 3). There was little intercartilaginous ground substance present. The single cartilage cell row was composed of 12 or 13 columnar and 4 or 5 hypertrophic cells. The standard area of the metaphysis contained 18 thick and long spicules covered by large mitotically proliferating osteoblasts. The cortex measured 235 microns. The endosteum was again composed of large osteoblasts, and the bone marrow was completely regenerated. After 10 days, the epiphyseal disc was 400 microns high. The single cartilage cell row contained 13 or 14 columnar and 5 large hypertrophic cells. There were 27 thick interlacing trabeculae in the standard area. The cortex was 265 microns thick. Endosteum and periosteum were not remarkable. The intensification of ossification was even more pronounced in the female than in the male. Gradually, the growth processes declined and assumed a normal rate in both sexes. After 21 or 30 days of refeeding, the histological appearance of the skeletal tissues did not differ from that of the normal controls of corresponding sex and age.

*Comment.* In vitamin B complex deficiency, skeletal development continues for some time, although at a reduced rate. The vitamin reserves of the organism are apparently sufficient to permit these processes to go on for a time. The subsequent cessation of growth and the serous atrophy of the bone marrow are findings typical of inanition. They suggest that the food ingested was not utilized, probably because the vitamin reserves had become exhausted. The severity of these changes manifests itself when they are compared with those obtained previously in underfed growing guinea pigs[2] and rats.[3] In animals kept on a quantitatively restricted but adequately balanced diet, skeletal growth proceeded at a reduced rate, but it lasted longer than usual. Thus in rats allowed to gain only 5 g every 50 days throughout their life, the long bones were only 13 to 18 % shorter than in normally fed animals.[3] In other words not unless the utilizable food energy is at a minimum and probably incompatible with life, can the inherent growth tendency of the skeleton be completely suppressed. The lack of thiamine in the diet may be responsible for loss of appetite and consequently decreased intake of food. However, the advanced skeletal changes seen in vitamin B complex-depleted rats were out of proportion to the diminished intake of food and must thus be attributed to the lack of the vitamin B complex. Again, the male responded to the vitamin deficiency more readily than the female. At this early age, the growth rate of the male is higher than that of the female. Any factor injurious to growth must, under otherwise identical conditions, necessarily affect the former more severely than the latter. If refeeding was begun before the animals were moribund, skeletal growth was quickly resumed, surpassed temporarily the normal rate and finally returned to normal. These findings are in agreement with observations in the skeleton of undernourished and refed animals.[2-5]

The present findings in vitamin B complex-depleted rats were similar to but more marked than those seen in mice deficient in pantothenic acid, or pyridoxine.[6] Lack of riboflavin, on the other hand, inhibited growth less but produced more degenerative changes in the epiphyseal cartilage than total B complex deficiency.[7] Serous atrophy of the bone marrow was not observed if only one of the aforementioned components of the vitamin B complex was lacking in the diet.

Investigations are in progress to determine whether the changes seen in complete vitamin B complex deficiency represent a cumulative effect of all the constituents of the complex; or whether in the absence of one of the constituents the others, even if present in the diet, cannot be properly utilized. The latter condition might then be comparable to that shown to exist relative to the essential amino acids.[8]

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[2] Silberberg, M., and Silberberg, R., *Arch. Path.*, 1940, **30**, 675.

[3] Saxton, J. A., Jr., and Silberberg, M., *Am. J. Anat.*, 1947, **81**, 455.

[4] Jackson, C. M., *The Effects of Inanition and Malnutrition upon Growth and Structure*, P. Blakiston's Son & Co., Philadelphia, 1925.

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, **18**, 95; 1915, **23**, 439.

[6] Silberberg, R., and Levy, B. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, in press.

[7] Levy, B. M., and Silberberg, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 355.

*Summary.* In growing vitamin B complex-deficient rats, there is retardation and, finally, cessation of growth of cartilage and bone associated with serous atrophy of the bone marrow. The male is more sensitive to the deficiency than the female. After refeeding a stock diet, skeletal growth is resumed at an intensified rate resulting in early restoration of normal conditions.

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[8] Cannon, P. R., Steffee, C. H., Frazier, L. J., Rowley, D. A., and Stepto, R. C., *Fed. Proc.*, 1947, **6**, 390.

## 16246 P

## Toxicity of Equine Serum Treated by Alkali.

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Observations on the removal of antigenicity of proteins by alkali were early made by Wells,[1] TenBroeck[2] and have been confirmed and extended by a number of later workers. Recently, Davis and Eaton,[3] as well as Kazal, DeFalco and Arrow,[4] paid special attention to the toxicity of the resulting products but came to apparently conflicting conclusions. The former found alkali-treated bovine serum albumin to be non-toxic for white mice, rabbits, and for 2 dogs when given intravenously. On the other hand, Kazal *et al.* confirmed an earlier report by Lewis[5] that purified bovine plasma protein treated by sodium hydroxide gained acute toxicity for guinea pigs as soon as its antigenicity was lost. In view of the importance of these findings especially in relation to possible practical application, it seems worthwhile to make a similar study on horse serum treated by alkali and to observe its toxicity in rabbits, guinea pigs, and white mice. The results obtained are herewith presented.

After several preliminary trials, it was found that one-quarter normal solution of sodium hydroxide would, after a period of 30 days at 37°C, render normal horse serum so altered in antigenicity that rabbits immunized with the treated serum produced no precipitin against both normal and treated sera, gave occasional complement fixation reaction with either, and in half of these immunized animals, skin allergy to normal serum alone was demonstrated. Furthermore, of 10 guinea pigs sensitized to treated serum, only 3 out of 7 reacted mildly with normal, and 4 out of 6 similarly to alkali-treated serum without a single death. At the same time, 4 controls died when shocked with normal serum. Nine rabbits tolerated as much as one gram of treated serum protein per kilo body weight without any symptoms, whereas only 5 out of 12 immunized rabbits reacted with an elevation of body temperature to half a degree Centigrade following intravenous injections of 20 ml (800 mg protein) of the treated serum. Among 26 white mice weighing 20 g given intravenously 0.5 ml of the same (20 mg protein), 2 died in 6 hours, and one died in a week. The rest survived 4 weeks, the period of observation. However, among 9 guinea pigs weighing 200-250 g, only 2 out of 3 receiving less than 100 mg of treated serum protein intracardially survived, while all 6 receiving 120-240 mg of the same died almost immediately, following intracardial injection, from shock symptoms not unlike those described by Kazal *et al.*

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[1] Wells, H. G., *J. Inf. Dis.*, 1909, **6**, 506.

[2] TenBroeck, C., *J. Biol. Chem.*, 1914, **17**, 369.

[3] Davis, H. A., and Eaton, A. G., PROC. SOC. EXP. BIOL. AND MED., 1942, **59**, 246.

[4] Kazal, L. A., DeFalco, R. J., and Arrow, L. E., *J. Immunol.*, 1946, **54**, 245.

[5] Lewis, J. H., *Science*, 1943, **98**, 371 (quoted in [4]).

Thus it seems clear that, as in the case of alkali-treated bovine serum, alkali treatment of equine serum rendered it practically non-antigenic, but definitely toxic for guinea pigs. At the same time, it remained practically innocuous for rabbits and for white mice. The toxicity of treated serum for larger laboratory animals and the nature of this toxic substance will be the subjects of further investigation.

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## 16247 P

# Treatment of Euthyroid Cardiac Patients by Producing Myxedema with Radioactive Iodine.*

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Significant clinical improvement in euthyroid cardiac patients following total thyroidectomy has been reported.[1] The rationale of this procedure was verified in that the degree of alleviation of angina pectoris and congestive failure was generally proportional to the degree of reduction in the metabolic rate.[2] The advent of radioactive iodine has raised the possibility of achieving the same result.

*Abstract of Cases. Case 1.* EM, 46-year-old woman, entered the hospital in 1941 because of progressive ankle edema, dyspnea and orthopnea during the previous year. She had chorea at the age of ten. Physical examination revealed rheumatic heart disease, cardiac enlargement, mitral and aortic stenosis and insufficiency, auricular fibrillation, congestion of the lungs and liver, ankle edema and orthopnea. She improved after one week in the hospital. In 1942, symptoms and signs of thyrotoxicosis appeared; the basal metabolic rate was approximately plus 33%. A maximal subtotal thyroidectomy was done in 1943. Myxedema developed following this procedure; the signs and symptoms of congestive heart failure markedly improved and she returned to work. Thyroid extract, 0.065 g, was administered daily until January, 1947, when, for the fifth time, the patient entered the hospital with orthopnea, pleural effusion, ascites, and peripheral edema. Thyroid medication was omitted. Despite adequate digitalization, mercurial diuretics, and salt and water restriction, little improvement resulted during 3 weeks of treatment. Following discharge from the hospital, the above signs and symptoms became progressively worse, paroxysmal nocturnal dyspnea appeared, and she again entered the hospital on June 14, 1947. Slight improvement resulted from the above regime of treatment. The basal metabolic rate averaged approximately plus 5%, the serum cholesterol was 220 mg/100 cc. On July, 5, '47, 5 millicuries $I^{131}$ were administered. The total 3-day urinary excretion was 71%. Approximately 10 weeks later the basal metabolic rate was minus 14% and the serum cholesterol 400 mg %. On November 1, '47, 13.6 millicuries $I^{131}$ were administered. A tracer study on December 4, 1947, showed

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† The authors wish to acknowledge the helpful advice of Dr. Arthur K. Solomon of the Department of Biophysics, Harvard Medical School, and of Mr. Leonidas Marinelli of the Memorial Hospital, New York City.

[1] Blumgart, H. L., Levine, S. A., and Berlin, D. D., *Arch. Int. Med.*, 1933, **51**, 866.

[2] a. Blumgart, H. L., Berlin, D. D., Davis, D., Riseman, J. E. F., and Weinstein, A. A., *J. A. M. A.*, 1935, **104**, 17; b. Blumgart, H. L., Riseman, J. E. F., Davis, D., and Weinstein, A. A., *Am. Heart J.*, 1935, **10**, 596.

84% excretion in 3 days. On December 10, '47, 6 weeks after the second dose, the basal metabolic rate was minus 26% and the serum cholesterol 440 mg %. Clinical evidence of myxedema, including lethargy, hoarseness, dry hacking cough, cold dry yellow skin were present. Her cardiac status improved. She was able to sleep flat in bed and undertake considerably more effort without dyspnea. Peripheral edema reaccumulate more slowly and, therefore, mercurial diuretics were required less frequently. Thyroid extract, 6 mg per day, mitigated the symptoms of myxedema and has enabled the patient to maintain clinical improvement.

*Case 2.* 60-year-old man entered the hospital for the fifth time on June, 1947, because of progressive cyanosis, dyspnea, orthopnea, hydrothorax, and nightly attacks of acute paroxysmal dyspnea. He had been treated in the out-patient department and wards for more than 5 years. The clinical diagnoses were arterial hypertension, rheumatic heart disease, mitral and aortic stenosis and insufficiency, marked cardiac enlargement, auricular fibrillation, arteriosclerosis of the coronary and peripheral arteries, and congestive heart failure. Despite treatment including optimal digitalization, mercurial diuretics, restricted fluid and salt intake, and curtailment of activity, his clinical status became progressively worse. Following admission to the hospital, treatment, including the removal of 1700 cc from the right chest which had accumulated in one week since previous thoracentesis, resulted in slight improvement, but he continued to have paroxysmal dyspnea and was considered critically ill. Basal metabolic rates ranged from plus 8 to minus 8% and the serum cholesterol level was 200 mg %. Ten millicuries $I^{131}$ were administered on July 5, 1947. The total 3-day urinary excretion was 51%. On September 18, 1947, an additional 2.25 millicuries of $I^{131}$ were administered. Four weeks later, or 3½ months following the first dose, the basal metabolic rate had declined to minus 25% and the serum cholesterol increased to 600 mg %. Coincidentally, the clinical status of this patient improved. He was able to sleep uninterruptedly flat in bed and dyspnea was much less, despite increased activity up and about on the ward. Reaccumulation of fluid has been slower and he requires fewer injections of mercurial diuretics.

*Discussion.* In both patients, the uptakes of tracer doses of $I^{131}$ were consistent with results obtained in subjects with normal thyroid glands. The normal basal metabolic rates, the normal serum cholesterol values, the normal cardiac rates and the absence of any clinical evidences of thyrotoxicosis were consistent with the normal thyroid state. The elevation of the serum cholesterol values in reciprocal relation to the fall in the basal metabolic rates which occurred gradually as the clinical evidences of myxedema appeared, demonstrate that radioactive iodine produced hypothyroidism in these two patients. The clinical improvement concomitant with reduction in metabolic rate observed in these two patients was similar to that observed in previous studies of cardiac patients following surgical total thyroidectomy.[2]

Considerable attention was given to possibly toxic effects of the rather large doses of radioiodine administered to these patients. Our studies have failed to disclose such evidence. The optimum dosage and regime of administration of $I^{131}$, the regularity with which myxedema may be induced in such patients, the possible utilization of adjuncts, such as thiourea derivatives and thyroid stimulating hormone of the pituitary, and final appraisal of the clinical value of this procedure, are matters under investigation which prolonged and extensive studies and observations will clarify.

*Summary.* Radioactive iodine 131 was administered to 2 severely ill cardiac patients who showed no clinical or laboratory evidences of thyrotoxicosis or myxedema. In both instances, decided reduction in the metabolic rate coincided with clinical improvement. These results justify further studies and observations.

## 16248 P

# Insulin Hypoglycemia in Chick Embryos.*

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Landauer[1,2] has found that certain morphological abnormalities may be produced by injecting insulin into the yolk of 0-5-day chick embryos. In the course of an investigation into the carbohydrate metabolism of insulin-treated embryos we have found that a persistent hypoglycemia is produced by insulin applied in this manner. This phenomenon is interesting because the hormones usually related to insulin activity are probably not present during the first 7-8 days of embryonic life.

The only certain information about the pituitary gland concerns the onset of cytological differentiation. Rahn[3] has shown that acidophiles and basophiles do not appear before the 10th day (though both are preceded by a general basophilic tendency starting on the 8th day). The adrenal cortex (Okuda[4]) is not formed until the 6th day; when cortin first appears is uncertain. Islets of Langerhans are not differentiated until 8-9 days and detectable amounts of insulin do not appear until the 11th day. Needham[5] has emphasized that there is no significant glycolytic system in embryonic chick tissues during the earlier stages.

Two units of insulin (Iletin, Lillie) were injected into the yolk sac of 5-day embryos. Blood was obtained directly from the extra embryonic blood system by means of suction through micro cannulae inserted into the vessels. Different groups of embryos were bled on successive days following the injection. Analyses for sugar were made on .01 ml samples with a modified Folin-Malmros technic. The average error for 45 samples of a standard (100 mg % glucose) solution with this procedure was 5.4 mg; standard error was 8.8 mg. The data are presented in Table I. It can be seen that the hypoglycemia is pronounced 24 hours after the injection and that there is a recovery from the insulin effect some time between the 12th and 14th days (7-9 days after injection). Various authors have claimed that normally the embryo's insulin becomes an important factor in control of blood sugar level at this time (Needham[6]).

There is some evidence that a hypoglycemia may be produced at still earlier stages. Insulin (2 units) was injected into the yolk sac of 30-hour embryos. Blood samples were taken 2 days later. At this stage it was necessary to pool blood from two embryos to obtain a .01 mg sample. The average blood sugar value for 4 such samples was 106.9 mg % while that for controls was 145.7 mg %.

TABLE I.
Average of Blood Sugar Values in mg %: 2 units of insulin injected at 5 days.
(No. of cases in parentheses.)

| Days of incubation | 6 | 7 | 8 | 9 | 10 | 12 | 14 | 16 |
|---|---|---|---|---|---|---|---|---|
| Uninjected controls | 135<br>(11) | 145<br>(8) | 145<br>(8) | 147<br>(6) | 134<br>(9) | 146<br>(6) | 160<br>(10) | 157<br>(5) |
| Insulin treated | 80<br>(16) | 70<br>(10) | 76<br>(8) | 49<br>(6) | 58<br>(8) | 53<br>(5) | 135<br>(8) | 141<br>(3) |

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* This work was supported, in part, by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

[1] Landauer, W., *J. Exp. Zool.*, 1945, **98**, 65.

[2] Landauer, W., *J. Exp. Zool.*, 1947, **105**, 145.

[3] Rahn, H. J., *Morph.*, 1939, **64**, 483.

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[6] Needham, J., *Chemical Embryology*, 1931.

16249

# Total Serum Protein Concentration and Total Weight of Circulating Protein in the Rat.

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The relation of total serum protein concentration and total weight of circulating protein to body size and sex were studied in the rat. The animals were used concurrently for estimation of blood and plasma volumes reported previously.[1]

*Methods.* A total of 125 normal rats were used, 47 females and 78 males, ranging in weight from 48 to 308 g. The rats were divided into groups, each of approximately 10 animals. In each group, the weight range was less than 10% of the mean weight, in most cases considerably less.

Blood and plasma volumes were determined by the hemoglobin dye method previously described.[1] Serum from the rats in each group was pooled in aliquot portions, and the total serum protein concentration was measured by a modification of Kingsley's biuret method.[2]

Because hemoglobin was used in determining plasma volumes, it was necessary to correct the protein determinations. This was done by subtracting from the total circulating protein value the weight of hemoglobin injected, and by subtracting from the total serum protein concentration the calculated concentration of hemoglobin. The weight of hemoglobin injected was determined by the colorimetric method of Evelyn and Malloy.[3]

In this paper, the previously reported individual values for blood and plasma volumes of the rats within each group were averaged. The mean values of these groups were related to body surface area, calculated from the formula of Carman and Mitchell.[4]

*Results.* The blood and plasma volumes (Fig. 1), total serum protein concentration (Fig. 2), and total circulating serum protein (Fig. 3) were plotted against body surface area. The results fitted a straight line reasonably well in each category. Regression lines

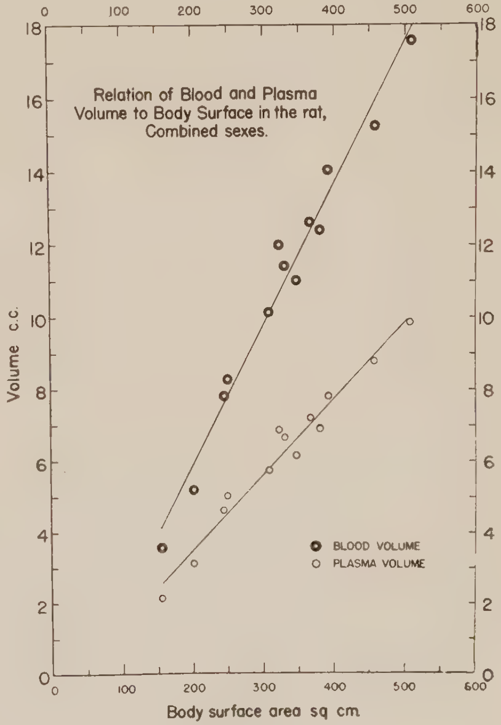

FIG. 1.

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[1] Lippman, R. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 188.

[2] Kingsley, G. R., *J. Biol. Chem.*, 1939, **131**, 197.

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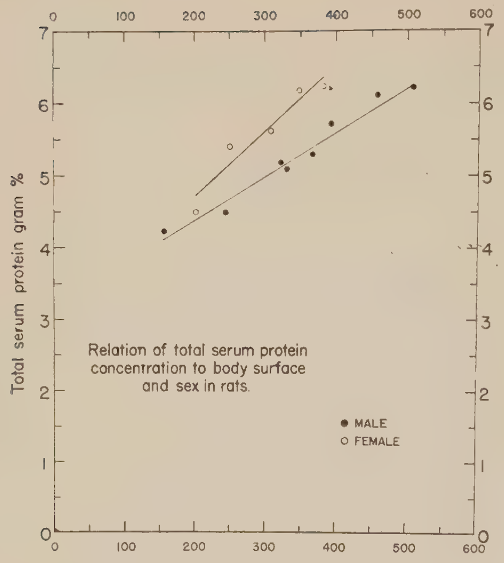

FIG. 2.

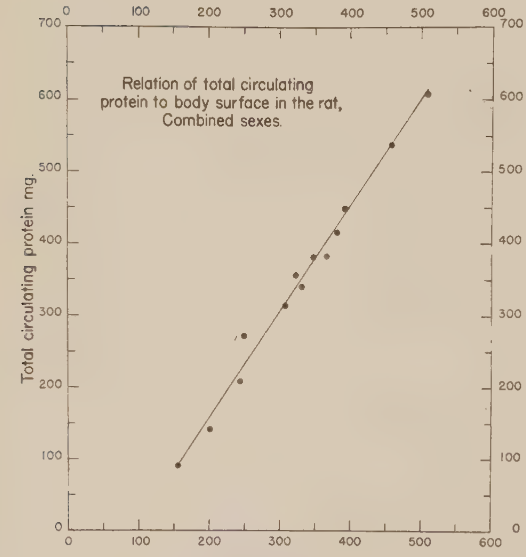

FIG. 3.

were calculated and the constants are given in Table I. There were slight sex differences in the regression constants for blood and plasma volumes and for total circulating protein, but as these did not appear significant for the purposes of this paper, the data were combined and are given in a single regression line. A significant sex difference in total serum protein concentration was found, and 2 regression lines are presented (Fig. 2).

*Discussion.* Few previous studies of the total serum protein concentration or the weight of total circulating serum protein in the rat have been made. Comparison is difficult, as different methods have been used to express results.

Cutting and Cutter[5] determined circulating plasma protein and plasma protein concentrations in rats. Their data were not segregated by sex, and they divided the rats into only 2 groups: "large" (292-395 g) and "small" (188-213 g). Their results, given in Table II, demonstrate a difference in the plasma protein concentration between the 2 groups. In addition, they found that the total circulating plasma protein in the large adult rats was greater in terms of surface area than in the smaller adult rats. Because female rats of the colony used by Cutting and Cutter rarely grow to weights over 300 g, it may be assumed that their "large" group consisted of male rats.

Metcoff and Favour[6] determined the total plasma protein concentration and total circulating plasma protein of rats, and likewise did not segregate their data by sex. They divided the rats into 3 groups: adult (119-335 g), pubescent (73-89 g), and prepubescent (40-68 g). Their total plasma protein concentrations were somewhat higher than other values reported, and they attributed this difference to the copper sulfate method of determination, which they used. However, their range of difference in total plasma protein concentration, between small rats and large rats, is similar to that which we found. They also found that the total circulating plasma protein increases in terms of body surface up to puberty, after which it remains relatively constant.

Our data, segregated by sex and into narrow weight ranges, appear to demonstrate a constant relationship of total circulating serum protein and serum protein concentration to body surface area over a wide range of weight which includes the range of puberty.

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[5] Cutting, W. C., and Cutter, R. D., *Am. J. Physiol.*, 1935, **113**, 150.

[6] Metcoff, J., and Favour, C. B., *Am. J. Physiol.*, 1944, **142**, 94.

TABLE I.
Relation of Blood Volume, Plasma Volume, Total Circulating Serum Protein, and Total Serum Protein Concentration to Body Surface Area.
General equation: $f(x) = A + Bx$
where x is body surface area in sq cm.

| f(x) | Sex | A | B |
|---|---|---|---|
| Blood volume (cc) | ♂ | — 1.59 | 0.0385 ± .0032 |
| | ♀ | — 1.90 | 0.0380 ± .0048 |
| | Combined | — 1.91 | 0.0390 ± .0016 |
| Plasma volume (cc) | ♂ | — 0.570 | 0.0211 ± .0026 |
| | ♀ | — 0.316 | 0.0192 ± .0026 |
| | Combined | — 0.710 | 0.0211 ± .0008 |
| Total circ. protein (mg) | ♂ | —150. | 1.50 ± .04 |
| | ♀ | —147. | 1.54 ± .06 |
| | Combined | —142. | 1.49 ± .04 |
| Serum protein conc. (g %) | ♂ | 3.15 | 0.00617 ± .0024 |
| | ♀ | 2.85 | 0.00920 ± .0026 |

TABLE II.
Previously Reported Values for Plasma Protein Concentration and Weight of Total Circulating Plasma Protein.

| Author | Wt group | Total plasma protein conc. g % | Total circ. plasma prot. mg/100 sq cm |
|---|---|---|---|
| Cutting and Cutter | Large (292-395 g) | 6.88 | 108 |
| | Small (188-213 g) | 6.30 | 82 |
| Metcoff and Favour | Adult (119-335 g) | 8.07 | 190 |
| | Pubescent (73-89 g) | 6.60 | 172 |
| | Prepubescent (40-68 g) | 5.62 | 118 |

This apparent discrepancy between our observations and those previously reported may be explained by the fact that the regression lines do not intercept the x-axis at 0, so that the ratio $f(x)/x$ increases as x increases. Although the lines are valid over the observed range, the relationship must change in extremely young rats, as it must ultimately pass through the point of origin.

We have related our results to body surface area of the rat, although we realize the imperfections of body surface as a reference measure. Kleiber[7] has clearly discussed the errors of body surface area calculations and relates metabolic rate to the 0.75 power of body weight. Our blood and plasma volume data exhibit a closely similar relationship.[1] We have used the relation of variables to body surface area here because it affords some comparison with other discussions in the literature.

*Summary.* 1. The total circulating serum protein and the serum concentration of total protein in the rat are shown to be related to body surface area over a wide range. 2. The sex difference in total serum protein concentration is of sufficient magnitude to require its consideration in experiments.

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[7] Kleiber, M., *Physiol. Rev.*, 1947, **27**, 511.

### 16250

# Effects of Protein and Fluid Consumption Upon Plasma Volume and Circulating Protein in the Rat.

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In studying rats under experimental conditions, changes in the concentration of serum constituents were noted. It was considered possible that such changes might be the consequence of changes in blood and plasma volume, induced by alterations in the consumption of food and fluid, rather than the consequence of a direct metabolic effect. This study was undertaken in order to determine whether the conditions of our experiments had produced such alterations in blood and plasma volumes.

*Methods.* A total of 101 normal male rats were used, ranging in weight from 148 to 196 g. The rats were taken from stock, where they had received a diet containing 17% of protein, and water as desired. They were placed in individual cages and given experimental diets for a specified time. At the end of this time, blood and plasma volume determinations were made by the method previously reported.[1] The serum from each experimental group of 8 to 10 rats was pooled in aliquot portions, and the total serum protein was determined by a modification of Kingsley's biuret method.[2] Exact composition of the diets is given in Table I. Total circulating protein was calculated from the measured data.

The rats received unrestricted amounts of the 3% and 77% protein diets and water. With the exception of the total starvation group, each group received an adequate number of calories.

*Results.* Detailed results are given in Table II, and compared with normal control values previously established for rats of the same weight taken directly from stock diet.[3]

None of the protein-deficient diets, whether of high or low fluid content, induced any appreciable change in plasma volume at the end of 7 days, although the total blood volume was increased about 10% when compared with normal for the final weight.

The fluid diet with added salt appeared to increase both total blood volume and plasma volume about 10% at the end of 17 hours. The 77% protein diet produced a similar change at the end of 7 days.

Neither the fluid diet without salt, nor total starvation for 17 hours altered the total blood or plasma volume.

None of the 17-hour experiments showed any significant change in total circulating serum protein or total serum protein concentration. However, all the protein-deficient diets reduced the total circulating serum protein by 15-20% in 7 days, with an almost commensurate reduction in the total serum protein concentration. The 77% protein diet did not produce any significant change in total circulating serum protein or in the total serum protein concentration during the period studied.

*Discussion.* Although there have been many studies of the effect of diet upon total serum protein concentration, very few have correlated such effects with changes in the blood and plasma volumes, or have studied volume changes alone.

Boycott and Chisolm[4] found no change in blood volume, but a fall in plasma volume,

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* The author gratefully acknowledges the technical assistance of Helen J. Ureen and Way Lew. This work was aided by a grant from the U. S. Public Health Service. Hemoglobin solution was provided by Sharp and Dohme, Inc., Philadelphia, Pa. Dr. Lippman is now at the Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles, Calif.

[1] Lippman, R. W., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 188.

[2] Kingsley, G. R., *J. Biol. Chem.*, 1939, **131**, 197.

[3] Lippman, R. W., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 193.

TABLE I.
Percentage Composition of Diets.

| Constituent | Stock diet (17% prot.) | 3% protein | 77% protein | 10% dext. | 10% dext. with salt |
|---|---|---|---|---|---|
| Bone ash | 1.5 | | | | |
| Soy bean meal | 5.7 | | | | |
| Wheat | 73.4 | | | | |
| Corn starch | 4.0 | 74.0 | | | |
| Casein | 9.7 | | 74.0 | | |
| Yeast | | 10.0 | 10.0 | | |
| Sardine oil | 3.2 | 10.0 | 10.0 | | |
| Alfalfa | 2.0 | 2.0 | 2.0 | | |
| Salt mixture | 0.5 | 4.0 | 4.0 | | |
| NaCl | | | | | 0.4 |
| Vit. B complex | | | | 1.0 | 1.0 |
| Dextrose | | | | 10.0 | 10.0 |
| Water | | | | 89.0 | 88.6 |

TABLE II.
Effect of Various Regimens on Blood and Plasma Volume and Serum Protein.
Mean Results.

| Diet | Total starv. | 10% dext. | 10% dext. with salt | 10% dext. | 10% dext. with salt | 3% prot. | 77% prot. |
|---|---|---|---|---|---|---|---|
| Duration | 17 hr | 17 hr | 17 hr | 7 days | 7 days | 7 days | 7 days |
| No. rats | 9 | 16 | 20 | 8 | 8 | 20 | 20 |
| Initial wt, g | | | | 151 | 153 | 172 | 172 |
| Final wt, g | 147 | 173 | 163 | 119 | 125 | 155 | 185 |
| Blood vol., cc | 11.29 | 11.91 | 12.56 | 9.67 | 10.01 | 11.57 | 13.68 |
| % normal | 109 ± 1.4 | 99 ± 2.1 | 112 ± 1.1 | 110 ± 2.3 | 110 ± 2.3 | 107 ± 2.6 | 110 ± 2.3 |
| Plasma vol., cc | 6.22 | 6.66 | 7.14 | 4.90 | 5.35 | 6.10 | 7.52 |
| % normal | 103 ± 1.8 | 100 ± 2.6 | 111 ± 1.4 | 96 ± 3.4 | 103 ± 3.8 | 99 ± 2.5 | 106 ± 2.3 |
| Total serum protein, g % | 5.33 | 5.42 | 5.28 | 4.28 | 4.48 | 4.70 | 5.62 |
| % normal | 105 | 102 | 101 | 89 | 92 | 92 | 102 |
| Total circ. protein, mg | 332 | 361 | 377 | 210 | 240 | 287 | 423 |
| % normal | 102 | 96 | 106 | 81 | 87 | 84 | 106 |

after 9 to 11 days on a protein-free diet. Frisch, Mendel and Peters[5] produced marked depression of serum protein concentrations by using a carrot diet for 18 weeks. Bloomfield,[6] on the contrary, found that an otherwise balanced 3% protein diet did not produce much lowering of serum protein concentration at the end of 21 weeks.

Metcoff, Favour and Stare[7] studied protein deficiencies in the rat, and their conditions for "acute protein deficiency" closely resembled our 3% protein diet for 7 days. They found, under these conditions, a decrease in the circulating total protein per unit surface area, and in the plasma volume per unit surface area.

The apparent increase that we found in the cell volume of rats with protein deficiency is probably due to the stability of the erythrocyte mass compared with the lability of plasma volume and body weight. In one week, the erythrocyte mass formed at the starting weight would not have changed appreciably, while the plasma volume and body weight had diminished, so that the erythrocyte mass was relatively increased.

We have avoided using the relation of variables to body surface area in these comparisons because of the pitfalls that have

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[4] Boycott, A. E., and Chisolm, R. A., *J. Path. and Bact.*, 1911, **16**, 263.

[5] Frisch, R. A., Mendel, L. B., and Peters, J. P., *J. Biol. Chem.*, 1929, **84**, 167.

[6] Bloomfield, A. L., *J. Exp. Med.*, 1933, **57**, 705.

[7] Metcoff, J., Favour, C. B., and Stare, F. J., *J. Clin. Invest.*, 1945, **24**, 82.

been suggested previously.[3] The situation would be additionally complicated by the fact that body surface formulae are derived from rats on stock diet. It is known that, after starvation or over-feeding, the composition of the body changes with respect to the basic constituents such as protein.[8] With such changes, the relation between body weight and surface area may change. Therefore, we have compared our results in each group with rats on stock diet at the same weight.

*Summary.* 1. Drastic changes in protein, fluid, and salt consumption do not produce striking changes in blood and plasma volumes of the rat, over the periods studied. 2. Increased fluid consumption produced an increase of about 10% in total blood volume and plasma volume at the end of 17 hours, only when salt consumption was simultaneously increased. 3. A high protein diet increased blood and plasma volumes slightly at the end of 7 days, but did not appreciably alter the serum protein concentration. 4. All protein-deficient diets reduced the total circulating protein by 15-20% at the end of 7 days.

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[8] Addis, T., *Proc. 6th Pacific Science Congress*, 1939, **6**, 677.

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### 16251

## Effect of Lipotropic Agents in a Diet Containing Pure Amino Acids in Place of Protein.

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In an experiment where an amino acid mixture replaced casein in a diet deficient in lipotropic factors, Rose, Machella, and György[1] found no appreciable effect of methionine fed to rats at a level of 50 mg per day or equivalent to about 0.8% of the diet. It was not immediately possible to extend this work but the need to determine whether more methionine would be effective or whether some other factor was involved was recognized. In the present study the effect of methionine in the original and in larger dosage was compared with that of choline.

*Experimental.* The experimental diet and the procedure followed were as previously described. Male albino rats, weighing 150 to 165 g were divided into 4 groups of which one received basal diet alone, the others receiving in addition respectively, 50 mg of methionine, 100 mg of methionine and 25 mg of choline chloride per day in aqueous solution. For about three-fourths of the animals we followed our usual procedure of giving the supplement in a Caster Cup. Since some of the rats disliked the solution of methionine a stomach tube was used for supplementing those animals which started later in the experiment (equal numbers of all groups). The animals were sacrificed at the end of 3 weeks and the liver fat determined.

*Results and Discussion.* The experimental data are summarized in Table I. As previously observed[1] the basal diet was quite inadequate in maintaining the weight of the animals and addition of the sulfur containing amino acid neither improved the nutritive value of the ration in this regard to any great extent, nor encouraged appetite. Choline was equally without effect on these factors. Food intake was remarkably constant in all of the groups but much lower than in the previous experiment. The average consumption was 4.1 g per day as compared with 5.9 g in the earlier study.

In the control animals the low food intake and weight loss did not prevent the development of fatty livers, an average value of 20.4% being found. A greater effect of methionine was observed in this experiment than in that previously reported. There was

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* The authors are grateful to Miss Caroline Perna for technical assistance.

[1] Rose, C. S., Machella, T. E., and György, P., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 352.

TABLE I.
Effect on Body Weight, Food Intake, and Liver Fat of Adding Methionine and Choline to a Diet Free of Sulfur-Containing Amino Acids.

| Group | Special supplement (daily) | No. of rats | Weight of animals | | Food intake, g/day | Liver | |
|---|---|---|---|---|---|---|---|
| | | | Initial, g | Change, % | | Wt, g | Total lipid, % |
| I | None | 12 | 156<br>(150-164) | —32 ± 1.4 | 4.2 ± 0.18 | 4.7 ± 0.12 | 20.4 ± 1.6 |
| II | 50 mg Methionine | 12 | 155<br>(150-165) | —26 ± 1.2 | 4.1 ± 0.13 | 4.3 ± 0.15 | 13.3 ± 1.3 |
| III | 100 ” ” | 12 | 155<br>(150-160) | —27 ± 1.9 | 4.1 ± 0.17 | 4.2 ± 0.15 | 9.7 ± 0.75 |
| IV | 25 ” Choline | 12 | 154<br>(150-162) | —29 ± 1.1 | 4.0 ± 0.11 | 3.8 ± 0.12 | 6.8 ± 0.36 |

significant reduction of liver fat at the 50 mg level (average fat, 13.3%), with still lower values when 100 mg were given (average fat, 9.7%). This result, with the lower food intake and greater weight loss in this experiment, suggests that the 50 mg level of methionine was at the borderline of effective dosage, and in the present case, the higher ratio of methionine in the diet (1.2%) was sufficient to produce a lipotropic effect.

When choline was given it showed its usual efficient lipotropic action. The average liver fat content of the choline-fed group was 6.8%, an almost normal value. Methionine in the 100 mg dosage gave a 40% higher fat content of the liver. The total methyl in this amount of methionine is 25% greater than in the choline.

It may be noted that the weight of the livers paralleled the fat content consistently.

*Summary.* Rats fed an alipotropic diet in which a mixture of amino acids replaced protein developed fatty livers in spite of low food intake and emaciation. Methionine in adequate dosage exerted a lipotropic effect although there was still 10% of fat in the liver when the methionine constituted 2.4% of the diet. When 25 mg of choline chloride per day were fed the livers were essentially normal.

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### 16252

## Therapeutic Effectiveness of Single and Divided Doses of Penicillin in a Streptococcal Infection in Mice.

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In a preliminary study on penicillin dosage and its therapeutic effectiveness it was found that a single oral dose containing 10 mg of penicillin G per kg of body weight protected a large proportion of mice in an otherwise fatal experimental streptococcal infection.[1] The results of further studies on the effect of single doses of drug, administered orally or subcutaneously, are given in the present communication.

In addition, a comparison between the effect of a given amount of penicillin when administered as a single dose and as a series of equally spaced smaller doses has been included in this report. Such a comparison is of interest, since it is commonly assumed that the therapeutic effectiveness of penicillin in aqueous solution depends upon repeated

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[1] White, H. J., Lee, M. E., and Alverson, C., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 35.

TABLE I.

Single Oral and Subcutaneous Doses of Penicillin G.

Treatment: Penicillin G sample containing 650 units per mg; administered as a single dose immediately after infection; in 0.5 cc vol. of aqueous sol. by stomach tube, or in 0.2 cc vol. subcutaneously.

| Single dose* mg/kg | Oral Alive/Total† | Subcutaneous Alive/Total† |
|---|---|---|
| 25.6 | 30/30 | |
| 12.8 | 28/30 | |
| 6.4 | 24/30 | |
| 3.2 | 23/30 | 30/30 |
| 1.6 | 5/30 | 23/30 |
| 0.8 | 0/30 | 18/30 |
| 0.4 | | 7/30 |
| 0.2 | | 1/30 |
| 0.1 | | 0/30 |

| | Oral mg/kg | Subcutaneous mg/kg | Oral:Subcutaneous dosage ratio |
|---|---|---|---|
| Median survival dose<br>(19/20 limits of error)‡ | 2.9<br>(3.8-2.2) | 0.78<br>(0.99-0.62) | 3.7<br>(5.4-2.5) |

* Dose expressed as pure penicillin G containing 1667 units per mg.

† Survival on 21st day after infection, expressed as number of mice alive out of total number tested.

‡ Interpreted as meaning that the chances are 19 to 1 that the true values fall within the indicated limits.

injections at 2- or 3-hour intervals.[2]

*Methods.* Our experimental infection was produced by intraperitoneal inoculation of 0.5 cc of a $10^{-5}$ broth dilution of a 6-hour blood broth culture of *Hemolytic streptococcus,* Group A, strain C 203, in Vanderwerken white mice of 18-22 g weight. The number of organisms in the infecting dose averaged 4000 ± 1000; virulence titrations indicated that a single organism (or chain), as determined by colony count, constituted a lethal dose. In the present studies, 80 of 80 untreated control animals died within 48 hours after infection. Treated animals alive on the 21st day after infection are considered to be cured.[3]

Tests with penicillin G were carried out with the 650 unit per mg sample previously used;[1] crystalline penicillin X was kindly supplied by E. F. Williams of the Physics Division of these Laboratories. In each case, the penicillin was dissolved in sterile distilled water and the solutions were used without filtration.

*Results.* The results of single dose treatment with penicillin G are given in Table I. By use of a simplified method for the quantitative evaluation of dosage-response data, devised by Wilcoxon and Litchfield[4] and illustrated in Fig. 1, the Median Survival Dose ($SD_{50}$) for subcutaneous administration is estimated to be 0.78 mg per kg, with 19/20 limits of error of 0.99-0.62. The corresponding values for oral administration are 2.9 and 3.8-2.2 mg per kg. The ratio of oral to subcutaneous dosage for penicillin G is thus 3.7, with 19/20 limits of error of 5.4-2.5.

In Table II, results with single doses of penicillin X are given. In this case, it is evident that subcutaneous is about 8 times as effective, therapeutically, as oral administration.

The question of single, as against divided, dosage was investigated by comparing the therapeutic effectiveness of an amount of penicillin given according to each of 4 dosage schedules, as follows: (1) total amount as single dose immediately after infection (0 hr);

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[2] Dowling, H. F., Rotman-Kavka, G., Hussey, H., and Hirsh, H. L., *Am. J. Med. Sci.*, 1947, **213**, 413.

[3] Litchfield, J. T., Jr., White, H. J., and Marshall, E. K., Jr., *J. Pharm. and Exp. Therap.*, 1939, **67**, 437.

[4] Wilcoxon, F., and Litchfield, J. T., Jr., to be published.

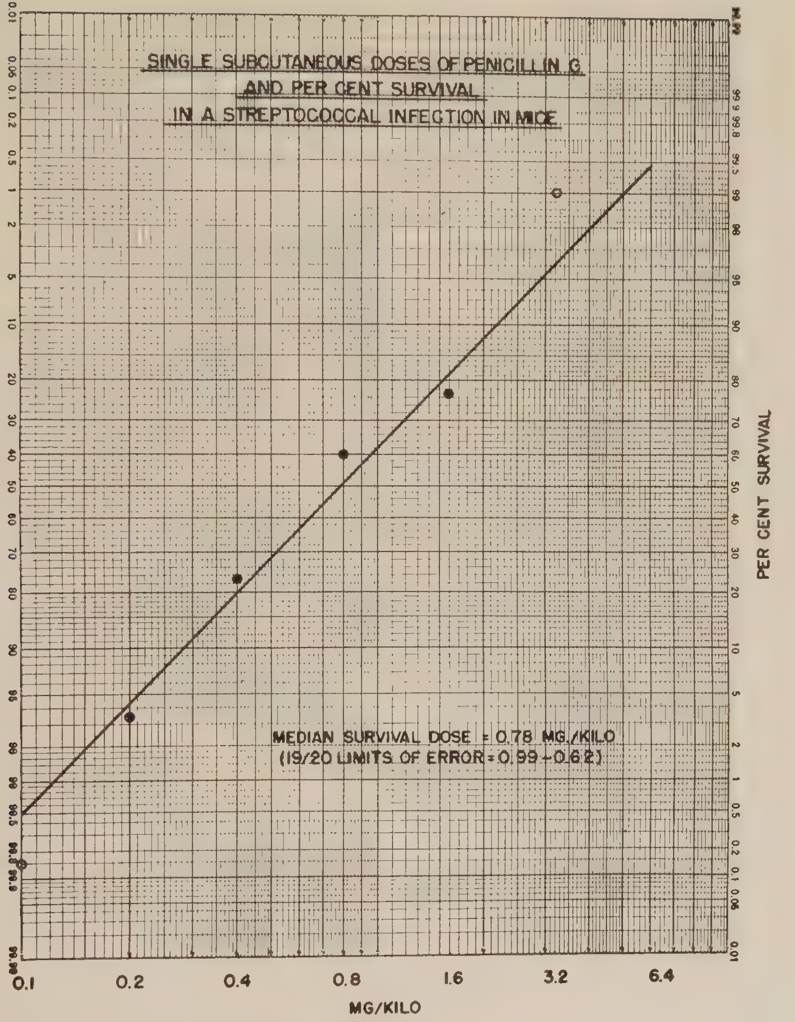

FIG. 1.
Single subcutaneous doses of penicillin G plotted against % survival on logarithmic probability paper.

TABLE II.
Single Oral and Subcutaneous Doses of Penicillin X.

Treatment: Crystalline penicillin X; administered as single dose immediately after infection; in 0.5 cc vol. of aqueous sol. by stomach tube, or in 0.2 cc vol. subcut.

| Single dose mg/kg | Orally Alive/Total* | Subcutaneously Alive/Total* |
|---|---|---|
| 16.0 | 10/10 | 10/10 |
| 8.0 | 9/10 | 10/10 |
| 4.0 | 8/10 | 10/10 |
| 2.0 | 4/10 | 10/10 |
| 1.0 | 1/10 | 9/10 |
| 0.5 | 0/10 | 8/10 |

* Survival on 21st day after infection, expressed as number of mice alive out of total number tested.

(2) ½ of total dose at 0 hour, the other ½, 12 hours later; (3) ¼ of total dose at 0 hour and ¼ each at 6, 12, and 18 hours later; (4) ⅛ of total dose at 0 hour and ⅛ each at 3, 6, 9, 12, 15, 18, and 21 hours later. These 4 schedules were used for each of 5 different total amounts of penicillin X, in one experiment, with 20 mice on each schedule for each amount, making a total of 20 dosage groups, each of 20 mice. Two separate experiments of this kind confirmed each other and the pooled results are given in Table III.

It is evident that no advantage was gained by splitting any amount of penicillin X into

TABLE III.
Single and Divided Doses of Penicillin X.
Treatment: Crystalline penicillin X; 0.2 cc vol. of aqueous sol. administered subcut. on dosage schedules indicated below; each dosage schedule began immediately after infection and was terminated within 24 hours.

| Amt of each dose mg/kg | Number of doses | | | |
|---|---|---|---|---|
| | One (At time of infection) | Two (At 12-hour intervals) | Four (At 6-hour intervals) | Eight (At 3-hour intervals) |
| 1.0 | 95* | ---- | ---- | ---- |
| 1/2 | 98 | 98 | ---- | ---- |
| 1/4 | 58 | 95 | 98 | ---- |
| 1/8 | 48 | 73 | 98 | 100 |
| 1/16 | 25 | 48 | 78 | 100 |
| 1/32 | ---- | 13 | 18 | 35 |
| 1/64 | ---- | ---- | 3 | 8 |
| 1/128 | ---- | ---- | ---- | 8 |

* Percentage survival on 21st day after infection; 40 mice were used on each of the 20 dosage schedules.

TABLE IV.
Median Survival Doses for Penicillin X Administered by 4 Different Schedules.

| Daily dosage schedule (Start: Immediately after infection) | | Median survival dosage* mg/kg | | |
|---|---|---|---|---|
| Frequency | No. of doses | Each dose | Total dose | 19/20 Limits of error† |
| Once | 1 | .13 | .13 | .43-.04 |
| q. 12 hr | 2 | .07 | .14 | .18-.11 |
| q. 6 hr | 4 | .04 | .16 | .22-.15 |
| q. 3 hr | 8 | .03 | .24 | .32-.21 |

* These values were obtained from dosage-response lines the slopes of which did not differ from each other significantly.
† The chances are 19 to 1 that the true median survival doses lie within these limits.

several small doses. For example, 8 doses of ⅛ mg per kg each were no more effective than a single dose of 1 mg per kg; and 8 doses of 1/64 mg per kg were not as effective as a single dose of ⅛ mg per kg.

As one would expect, 8 doses of ⅛ mg per kg each were more effective than 1 dose of ⅛ mg per kg; and, similarly, 8 doses of 1/16 each were more effective than a single dose of 1/16 mg per kg. However, multiple doses of ½ mg per kg could not improve on a single dose of ½ mg per kg since the maximum effect was obtained with the latter dose.

A quantitative comparison of the different dosage schedules may be based upon the Median Survival Dose for each schedule, as calculated from the dosage-response data in Table III. These values, together with their limits of error, are given in Table IV.

*Discussion.* The greater efficiency of subcutaneous over oral administration of penicillin, as measured by the relative amount of drug needed to produce the same degree of therapeutic effect (Tables I and II) confirms the well-known fact that about 5 times as much oral as subcutaneous dosage is needed to maintain a given concentration of the drug in the blood.[5,6] The choice between oral and parenteral dosage for clinical use would thus appear to depend upon such factors as relative cost and convenience to the patient.

The results obtained on single versus divided dosage (Tables III and IV) indicate that the total amount of penicillin X for a single day of treatment could be given in 1 or 2 doses, instead of 4 or 8 doses, without significant loss of protection. This is in agree-

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[5] McDermott, W., Bunn, P. A., Benoit, M., DuBois, R., and Reynolds, M. E., *J. Clin. Invest.*, 1946, **25**, 190.

[6] Hoffman, W. S., and Volini, I. F., *Am. J. Med. Sci.*, 1947, **213**, 513.

ment with the early work of MacLeod and Stone[7] who showed that mice infected with strains of pneumococcal types I, II, or III could be effectively treated with penicillin when the total daily dose (for each of 4 days) was restricted to only 3 injections within an 8-hour period. It is of interest to note, also, that Tillett, McCormack, and Cambier[8] have concluded that in the treatment of pneumococcal pneumonia with penicillin, repetition of injections continuously throughout each 24-hour period was in many cases not necessary or advantageous. Further experimental evidence indicating that there is no advantage in splitting daily penicillin dosage into a series of small frequently repeated doses has recently been reported by Zubrod[9] who investigated penicillins G and K in mice under conditions similar to those of the present report.

In the present investigation, the comparison of single and divided dosage schedules has been confined to a maximum treatment period of only 24 hours. That different results may be obtained under other experimental conditions, is indicated by the data of MacLeod and Stone.[7] For example, these workers found that treatment with penicillin for a 4-day period could be much more effective than giving an equivalent amount of drug for a single day. In mice infected with a type I pneumococcal strain, recovery of only 74% could be obtained with a total of 600 Oxford units when treatment was restricted to a single day, whereas, recovery of 100% resulted from treatment with a total of only 60 units divided into 4 daily amounts of 15 units each. With type II pneumococcus, about the same degree of recovery (60 to 70%) was produced by a total of 300 units, regardless of whether it was administered in a single day or divided into 4 daily amounts of 75 units each.

*Conclusion.* From 4 to 8 times as much penicillin was needed for oral, as for subcutaneous administration, to obtain the same degree of therapeutic effect in a streptococcal infection in mice.

In this infection, the administration of daily penicillin dosage in a series of small equally spaced doses was not more effective than giving an equivalent amount of drug in 1 or 2 doses per day.

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[7] MacLeod, C. M., and Stone, E. R., *Bull. N. Y. Acad. Med.*, 1945, **21**, 375.

[8] Tillett, W. S., McCormack, J. E., and Cambier, M. J., *J. Clin. Invest.*, 1945, **24**, 589.

[9] Zubrod, C. G., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 400.

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### 16253

## Effect of Passive Sensitization and Anaphylactic Shock on Rabbit Bone Marrow.*

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Concepts relating the plasma cell to hyperglobulinemia, globulin formation, immune mechanisms, hypersensitivity, and anaphylactic shock have a firm basis in both clinical and experimental literature.[1-10] Beginning with

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Maciesca-Jelenska, S., *Muench. Med. Wchnschr.*, 1907, **54**, 1790.

[2] Downey, H., *Folia haematol.*, 1911, **11**, 275.

[3] Huebschmann, P., *Verhandl. d. deutsch. path. Gesellsch.*, 1913, **16**, 110.

[4] Klein, *Z. f. d. ges. Neurol. u. Psychiat.*, 1914, **21**, 242.

[5] Oeller, H., *Deutsche. Med. Wchnschr.*, 1923, **49**, 1287.

[6] Siegmund, H., *Verhandl. d. deutsch. path. Gesellsch.*, 1923, **19**, 114.

[7] Epstein, E., *Virch. Arch. f. path. Anat.*, 1929, **273**, 89.

[8] Mas y Magro, F., *Arch. f. Exp. Zellforsch.*, 1929, **8**, 415.

the interest of Bing and Plum[11] in the relationship of bone marrow plasmacytosis to hyperglobulinemia there has accumulated a recent literature which clearly demonstrates that in man and animals reticular organ plasmacytosis occurs concomitant to the development of hyperglobulinemia in a great variety of conditions. Kolouch,[12,13] studying myelograms of animals sensitized to and shocked with bacterial antigens, discovered a cytomorphic cycle initiated by sensitization and fired by anaphylactic shock that led to bone marrow plasmacytosis. The development of plasma cells, from the plasmacellular reticulum of Rohr,[14] Kolouch found to be associated with rising serum antibody titer. Since this publication, a number of investigators have become interested in the plasma cell of reticular organs and experiments have been performed which indicate that this cell is the source of abnormal globulin and especially antibody globulin.[15-19] In a series of experiments Kolouch, Good, and Campbell[20] clarified the morphological mechanisms of reticular organ plasma cell genesis as previously pointed out by Kolouch and showed that the plasma cell content of bone marrow and spleen could be increased following active sensitization and shock with simple protein antigen (egg white) as well as with bacterial antigen. The phenomenon is one, then, of general hypersensitivity and shock and is not limited to bacterial antigens.

The possible relations of plasmacytosis to passive sensitization and shock have never been investigated. Bjoerneboe and Gormsen[17] have shown that passively induced hyperglobulinemia does not bring about reticular organ plasmacytosis. Bjoerneboe, Gormsen and Lundquist[21] as well as Fagraeus[18] also conducted experiments which seemed to indicate that increased plasma cell formation does not occur during heightened normal globulin formation. Further experiments carried out in our laboratory[22] indicate that local plasma cell formation, under some circumstances at least, is a function of tissue hypersensitivity and antigen-antibody interaction. In these experiments the plasma cells in the acute inflammatory reaction were shown to be a cytological accompaniment of hyperergic inflammation.

In order to test whether or not plasma cell formation is secondary either to passive sensitization and shock the following experiments were performed. Twenty-four young adult albino rabbits were actively sensitized to a simple protein antigen by injecting egg white on alternate days in the following dosages: 1 cc i.v., .5 cc i.v., and 1 cc i.m. A period of 18-30 days was then allowed to elapse before these rabbits were used as blood donors in the following experiment. Blood was obtained by cardiac puncture under aseptic precautions and allowed to clot. The antibody-rich serum was then separated carefully from the clot and that from several donors pooled to provide the serum used for the passive sensitization.

Six young adult albino rabbits weighing 4.0 to 5.3 lb were used for this experiment. Bone marrow biopsies were made after the

TABLE I.

| | | |
|---|---|---|
| No. 1055 | severe | shock |
| ” 1058 | moderate | ” |
| ” 1057 | mild | ” |
| ” 1061 | moderate | ” |
| ” 1062 | mild | ” |
| ” 1063 | no | ” |

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[9] Miller, F. R., *J. Exp. Med.*, 1931, **54**, 333.

[10] Perlzweig, W. A., Delrue, G., and Geschickter, C., *J. A. M. A.*, 1928, **90**, 755.

[11] Bing, J., and Plum, P., *Acta Med. Scandinav.*, 1937, **92**, 415.

[12] Kolouch, F., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 147.

[13] Kolouch, F., Thesis U. of M., 1938.

[14] Rohr, K., *Folia haematol.*, 1936, **55**, 305.

[15] Bjoerneboe, M., and Gormsen, H., *Nordisk Medicin*, 1941, **9**, 891.

[16] Bjoerneboe, M., and Gormsen, H., *Nordisk Medicin*, 1943, **19**, 1155.

[17] Bjoerneboe, M., and Gormsen, H., *Acta Path. et microbiol. Scand.*, 1943, **20**, 649.

[18] Fagraeus, A., *Nordisk Medicine*, 1944, **23**, 1580.

[19] Fagraeus, A., *Nordisk Medicin*, 1946, **30**, 1381.

[20] Kolouch, F., Good, R. A., and Campbell, B., *J. Lab. and Clin. Med.*, 1947, **32**, 749.

[21] Bjoerneboe, M., Gormsen, H., and Lundquist, Fr., *J. Immunol.*, 1947, **55**, 121.

[22] Good, R. A., unpublished M.S.

TABLE II.*

| No. 459 | Cells | Plasma cells | Plasmacellular reticulum cells | Reticulum cells |
|---|---|---|---|---|
| Effect of Active Sensitization and Shock on Bone Marrow. | | | | |
| Control | 1000 | 9.50 | 2 | 9.75 |
| Pre-shock | 1000 | 15.50 | 37.4 | 8.20 |
| 24 hrs post shock | 1000 | 34.20 | 10.5 | 9.20 |
| 4 days post shock | 1000 | 36.20 | 11.75 | 10.50 |
| Effect of Passive Sensitization and Shock on Bone Marrow. | | | | |
| No. 1058 | | | | |
| Control | 1000 | 5.25 | 1 | 9.50 |
| Pre-shock | 1000 | 9.0 | 2.5 | 10.2 |
| 24 hrs post shock | 1000 | 6.7 | 1.5 | 7.2 |
| 4 days post shock | 1000 | 6.2 | 2.5 | 1.0 |
| No. 1055 | | | | |
| Control | 1000 | 7.0 | 5.5 | 12.2 |
| Pre-shock | 1000 | 8.5 | 5.25 | 13.5 |
| 24 hrs post shock | 1000 | 6.5 | 2.75 | 14.5 |
| 4 days post shock | 1000 | 7.75 | 4.25 | 11.50 |

* Each figure is the mean of four 1000 cell counts.

method of Kolouch.[12] Each rabbit was given an injection of 15-30 cc of rabbit serum obtained from the actively sensitized rabbits described above. Eighteen hours later imprints were made of the bone marrow. Six hours later (24 hours after the injection of serum from the hypersensitive rabbits) each rabbit was injected with .4 cc egg white intravenously. All animals save one showed some degree of anaphylactic shock.

Each animal which showed shock was subjected to rib biopsy 8, 24, and 96 hours after the shock, and a large number of 1000-cell differential counts was made.

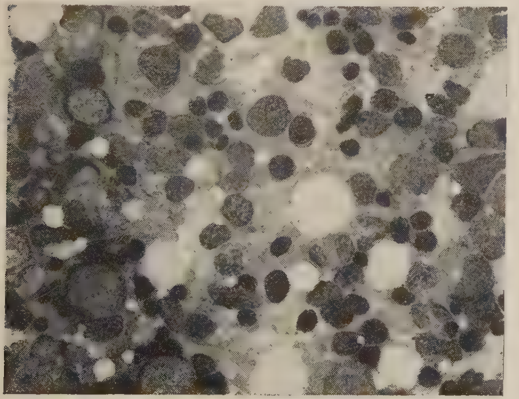

FIG. 1.

Bone marrow imprint from passively sensitized rabbit illustrating the presence of normal bone marrow. Note lack of plasma cells and plasmacellular reticulum cells. *Cf.* Kolouch, Good, and Campbell,[20] Fig. 2.

*Results.* The effect of passive sensitization and shock on bone marrow may be compared to that of active sensitization and shock in Table II.

While in active sensitization numerous plasmacellular reticulum cells flooded the bone marrow and following anaphylactic shock the bone marrow became filled with true Marschalko plasma cells, in passively sensitized and shocked rabbits no comparable changes were to be noted. The photo-micrograph, Fig. 1, illustrates this relationship. It is apparent that the cytomorphic cycle culminating in bone marrow plasmacytosis following active immunization or active sensitization and shock is due to factors other than reticular reaction secondary either to the presence of altered globulin in the form of antibody protein in the blood or tissues or factors related to the local or general effect of anaphylactic shock *per se*.

*Summary.* Studies of serial bone marrow biopsies of passively sensitized and shocked animals reveal: 1. Passive sensitization to a foreign protein does not activate the reticulum as does active sensitization. 2. Anaphylactic shock in passively sensitized rabbits is not followed by the development of bone marrow plasmacytosis as is the case with anaphylactic shock in actively sensitized animals.

16254 P

# Estimated Hepatic Blood Flow and Hepatic Venous Oxygen Content in Cirrhosis of the Liver.

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The hepatic blood flow in patients with cirrhosis is generally believed to be reduced because of destruction and distortion of the liver's vascular bed. Experiments designed to test this belief have been carried out by perfusing cirrhotic livers (obtained at necropsy) with saline solution or kerosene, but the results have been inconsistent. Working with large cirrhotic livers, Herrick[1] and Dock[2] concluded that the capacity for total blood flow in such livers is only moderately decreased and that the arterial inflow may even exceed the normal value. McIndoe,[3] who apparently used small, fibrotic livers, believed that hepatic blood flow is greatly reduced by increased resistance to flow in both hepatic arterial and portal venous systems. We have studied the problem in 14 patients with Laennec's cirrhosis, 11 of them with a history of chronic alcoholism, and 3 patients with cirrhosis due respectively to Schistosomiasis, syphilis, and chronic periportal inflammation (biliary cirrhosis).

*Methods.* Hepatic blood flow (EHBF) was estimated on the basis of the hepatic Bromsulfalein (BSP) clearance.[4] Hepatic venous blood samples were obtained by means of a venous catheter[5] placed in an hepatic vein of the right lobe of the liver under fluoroscopic control. Arterial blood was sampled simultaneously through an indwelling arterial needle from a brachial or femoral artery. Oxygen was determined in blood after the method of Van Slyke and Neill[6] and BSP was measured colorimetrically.[4] Oxygen concentrations were corrected on the basis of average arterial oxygen capacities. The subjects, selected from the wards of the Evans Memorial Hospital, Boston, and the Presbyterian Hospital, New York, were studied in the fasting basal state.

*Results and Comment.* Estimated hepatic blood flow was found definitely below normal (1000 to 2000 ml per minute) in 7 of 17 subjects, regardless of the etiology of the cirrhosis (Table I). In only 3 was EHBF significantly increased, whereas in 7 patients EHBF lay within the normal range.

The high values for EHBF obtained in K.M., B.M., and D.M. cannot be accepted without qualification, because the difference between the concentrations of BSP in hepatic and peripheral blood was so small that even a minor analytical error could have affected the EHBF significantly. Furthermore, in patients with severely damaged livers, the total amount of BSP removed by the liver may be over-estimated. In normal subjects, the removal is so efficient that any extra-hepatic removal is probably negligible, but in cirrhotics impairment of removal by the liver makes possible a proportionally greater removal extra-hepatically. Thus the error introduced by ascribing extra-hepatic removal of BSP to the liver would operate to yield falsely high values for EHBF. Hence, the preponderance of low values among the patients studied becomes more significant.

The data on oxygen content of hepatic venous blood is best discussed in terms of the arterio-venous difference. In all but 2 cases (see Table II) the oxygen A-V difference was greater than the normal difference between arterial and hepatic venous blood (2.5-4.5 ml %). Since only one of 5 subjects

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TABLE I.
Estimated Hepatic Blood Flow (EHBF) in Patients with Hepatic Cirrhosis.

| Subject | Age | Sex | Peripheral venous plasma concentration BSP, mg/100 cc | Change in peripheral venous concentration BSP, mg/100 cc/min. | Hepatic extraction* BSP, % | Total BSP removal, mg/min. | EHBF, ml/min. |
|---|---|---|---|---|---|---|---|
| Laennec's Cirrhosis, History of Chronic Alcoholism. | | | | | | | |
| I.C. | 46 | F | 3.74 | .000 | 12.0 | 2.39 | 850 |
| W.P. | 49 | M | 1.31 | +.005 | 22.2 | 1.46 | 876 |
| R.W. | 54 | M | 2.53 | —.005 | 18.7 | 2.20 | 932 |
| E.Mc. | 67 | F | 2.15 | +.005 | 15.8 | 1.85 | 983 |
| R.B. | 40 | M | 1.32 | +.006 | 36.4 | 2.76 | 990 |
| J.C. | 61 | M | 1.48 | +.005 | 21.4 | 2.72 | 1320 |
| L.F. | 56 | M | 0.86 | .000 | 24.0 | 2.16 | 1690 |
| F.F. | 43 | M | 0.48 | —.002 | 42.5 | 1.91 | 1735 |
| D.C. | 39 | F | 1.55 | +.010 | 10.3 | 1.85 | 1906 |
| O.H. | 38 | M | 1.09 | .000 | 22.9 | 2.54 | 1995 |
| D.M.† | 66 | M | 1.96 | .000 | 3.4 | 2.06 | 4020 |
| Laennec's Cirrhosis, Without History of Alcoholism. | | | | | | | |
| K.N.† | 38 | F | 1.85 | +.026 | 11.9 | 1.35 | 1026 |
| K.M. | 46 | F | 2.07 | +.003 | 5.5 | 1.87 | 2325 |
| B.M.† | 46 | M | 0.83 | +.002 | 8.4 | 2.28 | 4620 |
| Schistosomiasis—Periportal Fibrosis. | | | | | | | |
| M.D.† | 34 | M | 0.72 | .000 | 59.4 | 2.00 | 842 |
| Biliary Cirrhosis (Hanot's)—Periportal Fibrosis. | | | | | | | |
| F.G.† | 52 | F | 1.90 | +.010 | 12.1 | 1.45 | 1025 |
| Syphilitic Cirrhosis—Hepar Lobatum. | | | | | | | |
| J.L.† | 49 | M | 3.00 | .000 | 18.4 | 1.71 | 492 |

* Hepatic extraction of Bromsulfalein was calculated as the difference between peripheral and hepatic venous concentrations divided by the peripheral venous concentration.

† Diagnosis proved by biopsy or necropsy.

TABLE II.
Oxygen Content and Capacity of Peripheral Arterial and Hepatic Venous Blood in Patient with Hepatic Cirrhosis.

| | Arterial | | Hepatic venous | | Oxygen |
|---|---|---|---|---|---|
| Subject | Oxygen content, vol. % | Oxygen saturation, % | Oxygen content, vol. % | Oxygen saturation, % | Arterio-venous difference, vol. |
| Laennec's Cirrhosis with History of Chronic Alcoholism. | | | | | |
| A.A. | 14.0 | 89.6 | 8.3 | 52.6 | 5.7 |
| R.W. | 19.4 | 94.6 | 13.9 | 67.5 | 5.5 |
| F.F. | 17.4 | 95.0 | 12.0 | 65.2 | 5.4 |
| D.C. | 14.6 | 96.7 | 9.5 | 62.4 | 5.1 |
| D.M. | 7.3 | 84.5 | 6.0 | 69.0 | 1.3 |
| Laennec's Cirrhosis Without History of Alcoholism. | | | | | |
| K.N. | 15.9 | 93.4 | 12.5 | 73.2 | 3.4 |
| B.M. | 11.8 | 100.0 | 6.6 | 55.2 | 5.2 |
| Schistosomiasis—Periportal Fibrosis. | | | | | |
| M.D. | 14.8 | 96.0 | 7.8 | 50.0 | 7.0 |
| Biliary Cirrhosis (Hanot's)—Periportal Fibrosis. | | | | | |
| F.G. | 15.6 | 96.2 | 10.8 | 66.2 | 4.8 |
| Syphilitic Cirrhosis—Hepar Lobatum. | | | | | |
| J.L. | 14.3 | 95.9 | 8.6 | 57.1 | 5.7 |

with alcoholic cirrhosis exhibited a low A-V difference, our results do not support Dock's[2] suggestion that arterial flow tends to be greater in alcoholic than in non-alcoholic cirrhosis.

*Summary.* The hepatic blood flow tends to be low or normal in cirrhosis of the liver, regardless of etiology. The hepatic oxygen A-V difference is usually increased.

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## Antibiotic Studies on Beta Hemolytic Streptococci. I. Penicillin Resistance Acquired by Group A Organisms.

Horace M. Gezon. (Introduced by C. Phillip Miller.)

*From the Department of Pediatrics, University of Chicago.*

There are many reports on the development *in vitro* of resistance to penicillin by staphylococcus, pneumococcus, and meningococcus, but few on beta hemolytic streptococcus. Two groups of investigators studied a single strain of streptococcus (C203, a Group A, type 1 organism) and reported somewhat different rates of development of resistance. Rake and his co-workers[1] found a 30-fold increase in resistance after 58 transfers in penicillin broth, while Todd *et al.*[2] noted only a 5-fold increase after 35 subcultures and a 4-fold increase after 78 subcultures in penicillin broth. No references to induced resistance for other strains of Group A streptococci and none for Group B or C organisms were found.

The present study was made to look for differences among strains within the A Group. Those reported in the succeeding two communications were undertaken to compare these with the behavior of Group B and C organisms respectively. Variations in penicillin sensitivity have been shown by organisms of these 3 serological groups.[3-8] The similarities and differences reported by these investigators were observed by us in a screening test of 203 recently isolated strains of Group A, 16 strains of Group B, and 24 strains of Group C streptococci, using the Fleming[9] ditch plate method.

*Materials and Methods.* All the strains of beta hemolytic streptococci were isolated from cultures of the nose or throat obtained from patients with acute upper respiratory disease. None had received penicillin therapy. The organisms were grouped by the Lancefield technic. Bacto heart infusion agar (Difco), containing 5% defibrinated sheep's blood, was used as the solid medium. Veal infusion broth, containing 5% defibrinated sheep's blood, served as the liquid medium. In the earlier series, designated R and A, commercial, amorphous sodium penicillin (Lederle) was used, while in the later series, S, T, and U, crystalline sodium penicillin G (Lilly) was employed. Standardization of these penicillins and the final sensitivity tests were performed with standard sodium penicillin G* with a potency of 1667 units per mg.

The ditch plates used to determine the penicillin sensitivity of streptococci were prepared by removing a central trough from a blood agar plate and replacing it with melted agar containing 5% sheep's blood and varying concentrations of standard penicillin G. When this central strip solidified, heavy inocula of organisms were streaked in rows directly across the plate. The character of the growth on both penicillin and penicillin-free media was observed on these plates. The absolute penicillin sensitivity, however, could not be measured by this technic since penicillin diffused into the agar adjacent to the trough.

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[8] Rutherford, R. H., Marquardt, G. H., and Van Ravenswaay, A. C., *J. Missouri State Med. Assn.*, 1946, 535.

[9] Fleming, A., *Proc. Roy. Soc. Med.*, 1941, **34**, 342.

* Supplied through the courtesy of Dr. Henry Welch, Chief of the Division of Penicillin Control and Immunology, Food and Drug Administration.

TABLE I.
Penicillin Resistance Induced in Group A Beta Hemolytic Streptococci by Subculture on Penicillin Medium.

| Strain | Number of transfers | Initial penicillin Sensitivity,* u/ml | Final penicillin Sensitivity,* u/ml | Fold change |
|---|---|---|---|---|
| $S_1$ | 60 | .01 | .04 | 4 |
| $S_2$ | 60 | .02 | .04 | 2 |
| $S_3$ | 60 | .01 | .17 | 17 |
| $S_4$ | 60 | .01 | .01 | 0 |
| $S_5$ | 60 | .01 | .045 | 4.5 |
| $S_6$ | 60 | .01 | .04 | 4 |
| $U_5$ | 60 | .01 | .06 | 6 |
| $U_6$ | 60 | .01 | .17 | 17 |
| $A_1$ | 60 | .01 | .04 | 4 |
| $A_2$ | 60 | .01 | .04 | 4 |
| $R_2$ | 56 | $<.01$ | .015 | $>1.5$ |
| $R_4$ | 56 | $<.01$ | .02 | $>2$ |
| $R_5$ | 56 | $<.01$ | .03 | $>3$ |

* Penicillin sensitivity is defined as the highest concentration of penicillin in the medium which permits colonies to grow.

Penicillin sensitivity is defined as the maximum concentration of penicillin on which bacterial growth occurs on the medium in the trough.

To induce resistance, 5 to 7 strains of streptococci were inoculated in single lines on each blood agar plate of a series. Between 3 and 8 concentrations of penicillin were used in a set for each serial transfer. In the R series, subcultures were made alternately on plain blood infusion broth and blood agar medium containing penicillin for a total of 56 serial transfers on each. In the A, S, T, and U series the confluent bacterial growth on the blood agar plate was emulsified in saline and subcultured directly to a new set of plates; a total of 60 such subcultivations was made in each series. Transfers to the next set of plates were made from colonies on medium with the highest concentration of penicillin permitting growth. The penicillin sensitivity for any transfer was considered to be the highest concentration of penicillin that allowed good growth.

Mouse virulence titrations were made as follows: The organisms were incubated at 37°C for 18 hours in tryptose phosphate broth (Difco) containing 5% normal horse serum. The culture was centrifuged at 2000 RPM for 5 minutes, the supernatant fluid discarded, and the sediment resuspended in a sufficient quantity of normal saline to give a final density equal to the McFarland Standard No. 1. The average plate colony count with this concentration of organisms was approximately $2 \times 10^9$ organisms per ml of the saline suspension. Four- to five-week-old white mice (Germantown strain) were inoculated intracerebrally in pairs with 0.03 ml of the $10^0$ to $10^4$ dilution of the organisms. For this procedure the mice were lightly anesthetized with ether, the skin and hair sterilized with iodine, and the injection made midway between the right ear and eye at a depth of approximately 4 mm. The infected mice were observed for a period of five days. The $LD_{50}$ was calculated by the Reed and Muench formula.

A. *Resistance Acquired on Media Containing Penicillin.* Thirteen strains of Group A streptococci were subcultivated serially in an attempt to induce resistance to penicillin. A seventeen-fold rise in penicillin resistance was the maximum change obtained. In only two strains did an increase of this magnitude occur. Eleven organisms showed either no change or only a slightly increased resistance after 56 to 60 transfers onto media containing penicillin. Table I summarizes these results.

B. *Resistance Acquired on Control Media.* Eight sensitive parent strains of Group A streptococci were subcultured serially on penicillin-free blood agar medium. After 60 transfers no appreciable increase in penicillin resistance was found when tested by the penicillin whole plate method or by the ditch

TABLE II.
Comparison of the Rates of Development of Penicillin Resistance of Parent and Control Strains of Streptococci.

| Strain | Mean of first 15 transfers of parent* strain on penicillin medium u/ml | Mean of first 15 transfers of control† strain on penicillin medium u/ml |
|---|---|---|
| $S_1$ | .013 | .026 |
| $S_2$ | .015 | .012 |
| $S_3$ | .011 | .013 |
| $S_4$ | .012 | .017 |
| $S_5$ | .015 | .009 |
| $S_6$ | .011 | .011 |
| $U_5$ | .016 | .008 |
| $U_6$ | .015 | .019 |

* Parent strain is a freshly isolated organism.
† Control strain is the parent strain after 60 transfers on control medium.

plate method.

Because preliminary results suggested that adaptation of the streptococci to growth on artificial medium influenced the development of penicillin resistance, the organisms after 60 subcultures on control medium were changed to penicillin medium for 15 transfers. The mean value for the first 15 subcultures of the parent and the control strains on penicillin medium are given in Table II. From these values it is evident that repeated subculturing on plain blood agar does not facilitate the acquisition of resistance to penicillin for most strains of Group A streptococci.

C. *Loss of Acquired Resistance.* Five strains of streptococci after 56 to 60 transfers on penicillin agar, were subcultured serially on penicillin-free medium in an attempt to restore the original sensitivity. The three strains from the R series (R2, 4, and 5) were transferred daily 25 times and the 2 strains from the A series (A1 and 2), 50 times. At the end of 25 subcultures 4 of the 5 strains had returned to their original level of penicillin sensitivity. After 50 transfers the fifth strain had returned to its original level. Todd and his co-workers[2] observed this same behavior with a resistant culture of the C203 strain after 76 transfers in plain broth.

Four strains were passed serially by intracerebral inoculation in white mice. All of these streptococci had returned to their original level of penicillin sensitivity by the 12th passage.

D. *Change in Virulence.* Mouse virulence titrations were performed on 10 of the 13 parent strains, on subcultures of the same strains after 60 transfers on control medium, and on all strains after 56 to 60 transfers on penicillin medium. The results are summarized in Table III. All strains showed a marked loss of mouse virulence after serial transfers on penicillin medium. Most of the strains demonstrated relatively little loss of mouse virulence after serial transfers on control medium.

In an attempt to restore the virulence lost by growth on penicillin medium, five strains were passed intracerebrally 12 times through mice. An increase in virulence occurred in 3 of 5 strains.

E. *Antigenic, Hemolytic, and Colonial Changes.* The serological behavior of the streptococci after 56 to 60 serial transfers on penicillin or penicillin-free medium is given in Table IV. In 6 resistant and 2 control strains (*i. e.*, after repeated transfers on control medium) we were unable to demonstrate the presence of the group specific precipitinogen by the Lancefield technic.

A change in the hemolytic behavior of the streptococci when growing on penicillin medium was observed in 10 of the 13 strains studied. Transient conversion from beta to alpha or gamma type of colony appeared when the organisms were growing on maximal concentrations of penicillin. The same strains showed the normal beta hemolysis on lower concentrations of penicillin or on plain blood agar. In some instances a mixture of alpha, beta, and gamma types of colonies was evident in a given culture on the same concentration of penicillin. When single colonies of any of these types were picked under a dissecting microscope and subcultured on lower concentrations of penicillin or on plain blood agar, they always showed beta hemolysis. The possibility of contamination was eliminated by regrouping isolated alpha and gamma colonies.

The colonial appearance of resistant strains of streptococci did not differ appreciably from the parent or the control strains. How-

TABLE III.
Mouse Virulence of Group A Streptococci with Acquired Penicillin Resistance.

| Strain | Initial $LD_{50}$* | $LD_{50}$* after 60 transfers on | | $LD_{50}$* of resistant strain after 12 mouse passages |
|---|---|---|---|---|
| | | control medium | penicillin medium | |
| $S_1$ | .0000096 ml | .00012 ml | .0030 ml | .000048 ml |
| $S_2$ | .00012 | .00048 | .015 | .00096 |
| $S_3$ | .00096 | .000039 | >.03 | |
| $S_4$ | .000030 | .00075 | >.03 | |
| $S_5$ | .000096 | .00012 | .015 | |
| $S_6$ | .000030 | .000096 | >.03 | |
| $U_5$ | .00048 | .00019 | >.03 | |
| $U_6$ | .0012 | .00096 | >.03 | |
| $A_1$ | .00096 | .0096 | .0060 | |
| $A_2$ | .000030 | .0096 | .0096 | |
| $R_2$ | | | .030 | .0096 |
| $R_4$ | | | .030 | >.03 |
| $R_5$ | | | .030 | >.03 |

* $LD_{50}$ is stated as the amount of undiluted bacterial suspension in normal saline that will kill 50% of the mice injected. This suspension has a density equal to McFarland No. 1 standard and contains approximately $2 \times 10^9$ organisms per ml.

TABLE IV.
Group Specificity of Streptococci After 56 to 60 Serial Transfers on Penicillin and Penicillin-free Media.

| | Strain | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ | $S_6$ | $U_5$ | $U_6$ | $A_1$ | $A_2$ | $R_2$ | $R_4$ | $R_5$ |
| Parent culture | A | A | A | A | A | A | A | A | A | A | A | A | A |
| Final control culture | A | A | A | A | A | A | N | A | N | A | — | — | — |
| Final penicillin-resistant culture | A | A | A | A | A | A | N | N | A | N | N | N | N |

A = group A.
N = not groups A, B, or C.
— = grouping not performed.

ever, transient changes were noted during the process of serial transfers when organisms were growing on maximal concentrations of penicillin. On these plates the colonies frequently were tiny with narrow zones of alpha or beta hemolysis or with gamma hemolysis. No permanent dissociation from mucoid to matt or matt to glossy types was observed. When heavy inocula were streaked on plates with a concentration of penicillin too high to permit visible colonies, hemolysis in the medium and a disappearance of the surface luster were commonly found. Subcultures from these plates failed to grow.

*Discussion and Conclusions.* Penicillin resistance in strains of Group A streptococci would be of considerable clinical importance, but there is little evidence that such strains actually produce human infections. No proven instance has been found in a review of the literature. Experimentally, resistance to penicillin can be induced in most strains by *in vitro* technics. However, the increase in resistance, as compared with that of strains of staphylococci, is relatively slight with a 17-fold rise as the maximum change obtained. Using *in vivo* methods in embryonated eggs and in mice,[10] no alteration in penicillin sensitivity was observed. Furthermore, when resistance is induced, animal virulence falls. A similar loss of animal virulence has been described by Miller and Bohnhoff[11] for meningococci and McKee and Houck[1a,1b] for the C203 strain of Group A streptococcus, type 3 pneumococcus and staphylococcus.

The change in hemolysis seen in streptococci that are growing on medium containing penicillin may be the result of an alteration by penicillin in the metabolism of the organism. A change in streptolysin O or S must also be considered. Further study is necessary to elucidate the nature of this interesting phenomenon.

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[10] Unpublished data.

[11] Miller, C. P., and Bohnhoff, M., *J. Inf. Dis.*, 1947, **81**, 147.

*Summary.* 1. Thirteen strains of Group A streptococci were transferred serially on medium containing penicillin in an effort to induce resistance. Two strains developed a 17-fold increase in resistance. Eleven strains either remained unchanged or showed an increase up to only 6-fold at the end of 60 subcultures.

2. No increased resistance was induced in the same organism by serial transfers on control medium.

3. Strains which had acquired resistance were restored to their original level of penicillin sensitivity by serial subculture on penicillin-free medium or by serial intracerebral passages in mice.

4. A marked loss of virulence for mice was shown by all resistant strains of streptococci. The virulence was restored in 2 strains by passage in normal mice.

5. In about one-half of the resistant strains the group specific antigen could not be demonstrated.

6. Transient changes in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were shown by most strains when growing on maximal concentrations of penicillin.

I wish to acknowledge the valuable technical assistance given in these studies by Ernst Jaffe, Sylvia Simons, Deborah Patinkin, and Robert Goff.

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## Antibiotic Studies on Beta Hemolytic Streptococci: II. Penicillin Resistance Acquired by Group B Organisms.

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Most strains of group B beta hemolytic streptococci possess a higher degree of initial penicillin resistance than group A organisms. The range reported by 3 groups of investigators[1,2,3] was 0.0625 to 0.25 units of penicillin per ml of medium. We have found the upper and lower limits of sensitivity to extend from 0.01 to 0.10 unit of penicillin per ml of medium by the Fleming ditch-plate method[4] for 16 freshly isolated strains of group B streptococci.

The present study was carried out in an effort to determine the rate of development of penicillin resistance *in vitro*. In addition we were interested in learning whether the antigenic, hemolytic, colonial, and mouse virulence changes found in resistant group A organisms[5] occurred in group B streptococci.

*Results.* A. *Acquired Resistance on Medium Containing Penicillin.* Ten strains of group B streptococci were transferred on increasing concentrations of penicillin. The results are given in Table I. A straight line curve results when the mean of fifteen transfers is plotted on semi-logarithmic paper. The graph of the daily values shows the same general trend but with many irregularities in the curve. These variations can be explained by slight differences in the amount of medium per plate, daily loss of potency of penicillin in stock solution, and errors in judgment in estimating the proper range of penicillin concentrations needed for each transfer.

The maximum change occurred in the $R_1$

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[1] Watson, R. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 65.

[2] Simmons, R. T., and Christie, R., *M. J. Australia*, 1946, **1**, 349.

[3] Rutherford, R. H., Marquardt, G. A., and Van Ravenswaay, A. C., *J. Missouri State Med. Assn.*, 1946, 535.

[4] Fleming, A., *Proc. Roy. Soc. Med.*, 1941, **34**, 342.

[5] Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 207.

TABLE I.
Induced Penicillin Resistance in Group B Beta Hemolytic Streptococci.

| Strain | Number of passages | Initial penicillin sensitivity,* u/ml | Final penicillin sensitivity,* u/ml | Fold change |
|---|---|---|---|---|
| $T_1$ | 60 | .03 | .34 | 11 |
| $T_2$ | 60 | .03 | .65 | 22 |
| $T_3$ | 60 | .03 | .65 | 22 |
| $T_4$ | 60 | .03 | .55 | 18 |
| $T_5$ | 60 | .03 | 1.35 | 45 |
| $T_6$ | 60 | .03 | .65 | 22 |
| $T_7$ | 60 | .03 | .65 | 22 |
| $A_5$ | 60 | .015 | .90 | 60 |
| $A_6$ | 60 | .015 | .80 | 53 |
| $R_1$ | 56 | .01 | 1.90 | 190 |

* Penicillin sensitivity is defined as the highest concentration of penicillin in the medium which permits colonies to grow.

strain which developed a 190-fold increase in resistance in 56 transfers. However, between each transfer of this organism on penicillin medium it was subcultured in penicillin-free blood broth. With the other nine bacteria, the increase in penicillin resistance by the plate to plate technic varied from 11 to 60-fold in 60 serial transfers.

B. *Acquired Resistance on Control Media.* Seven strains subcultured from the original parent stock were transferred serially on penicillin-free blood agar plates. None of the group B organisms studied showed a change in penicillin sensitivity after 60 transfers, nor did they acquire resistance more rapidly than the parent strain when cultured subsequently on penicillin medium for the 61st to 75th passages. The results are given in Table II. This same behavior was described for Group A streptococci in the preceding communication.[5]

TABLE II.
Comparison of the Rates of Development of Penicillin Resistance of Parent and Control Strains of Streptococci.

| Strain | Mean of first 15 transfers of parent* strain on penicillin medium u/ml | Mean of first 15 transfers of control† strain on penicillin medium u/ml |
|---|---|---|
| $T_1$ | .088 | .072 |
| $T_2$ | .083 | .050 |
| $T_3$ | .120 | .051 |
| $T_4$ | .099 | .070 |
| $T_5$ | .105 | .076 |
| $T_6$ | .094 | .059 |
| $T_7$ | .100 | .070 |

* Parent strain is a freshly isolated organism.

† Control strain is the parent strain after 60 transfers on control medium.

C. *Loss of Acquired Resistance.* Three strains of streptococci, after acquiring penicillin resistance, were subcultured on plain blood agar. The $R_1$ strain was transferred 25 times, and the $A_5$ and $A_6$ strains 50 times. At the end of these serial subcultures there was no loss of the acquired resistance in any of the 3 organisms studied.

Three resistant organisms were passed serially by intracerebral inoculation in white mice for 12 passages. Again there was no loss of acquired resistance to penicillin.

*Change in Virulence.* Using the method described in the preceding communication,[5] the mouse virulence studies were performed on all 10 strains. The results are summarized in Table III. As with the group A organisms, all strains lost virulence after serial transfers on penicillin medium. The majority showed relatively little change after serial transfers on control medium.

Three resistant strains were passed 12 times through mice to restore the lost pathogenicity. The virulence reappeared in one strain. In 2 strains no significant change occurred.

*Antigenic, Hemolytic, and Pigment Changes.* The results of the serological grouping of the streptococci before and after serial transfers on penicillin and control media are summarized in Table IV. In 2 resistant and one control strain, it was not possible to demonstrate the presence of the group specific precipitinogen. A majority of the organisms in each classification remained groupable.

TABLE III.
Mouse Virulence of Group B Streptococci with Acquired Penicillin Resistance.

| Strain | Initial $LD_{50}$* | $LD_{50}$* after 60 transfers on control medium | $LD_{50}$* after 60 transfers on penicillin medium | $LD_{50}$* of resistant strain after 12 mouse passages |
|---|---|---|---|---|
| $T_1$ | .000048 ml | .0000015 ml | .0096 ml | |
| $T_2$ | $<$.0000030 | .000012 | $>$.03 | |
| $T_3$ | $<$.0000030 | .000060 | $>$.03 | |
| $T_4$ | .000096 | .00030 | .015 | $>$.03 ml |
| $T_5$ | $<$.0000030 | .0000000015 | $>$.03 | |
| $T_6$ | $<$.0000030 | .000075 | $>$.03 | |
| $T_7$ | $<$.0000030 | $>$.03 | .003 | .0048 |
| $A_5$ | .0015 | .00096 | $>$.03 | |
| $A_6$ | .012 | .0048 | .0096 | |
| $R_1$ | .00030 | | $>$.03 | .00048 |

*$LD_{50}$ is stated as the amount of undiluted bacterial suspension in normal saline that will kill 50% of the mice injected. This suspension has a density equal to McFarland No. 1 standard and contains approximately $2 \times 10^9$ organisms per ml.

TABLE IV.
Group Specificity of Streptococci after 56 to 60 Serial Transfers on Penicillin and Penicillin-Free Media.

| | Strain $T_1$ | $T_2$ | $T_3$ | $T_4$ | $T_5$ | $T_6$ | $T_7$ | $A_5$ | $A_6$ | $R_1$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Parent culture | B | B | B | B | B | B | B | B | B | B |
| Final control culture | B | B | B | B | B | B | B | N | B | — |
| Final penicillin-resistant culture | N | B | B | B | B | B | B | B | B | N |

B = group B.
N = not groups A, B, or C.
— = grouping not performed.

The alteration in the character of the hemolysis described for group A organisms in the preceding report[5] was even more striking and more permanent for group B streptococci. All 10 strains showed varying degrees of change from beta to alpha or gamma colonies at maximal concentrations of penicillin. Single colonies of either the gamma or alpha types when subcultured onto plain blood agar produced only beta hemolysis. Small colony forms occurred frequently on penicillin medium. These reverted back to the normal forms when transferred to control medium.

Several of the streptococci of Group B showed the presence of the reddish pigment described by Lancefield.[6] No modification in this pigmentation appeared after the organisms acquired penicillin resistance.

*Discussion and Conclusions.* All strains of Group B streptococci studied showed a rapid development of penicillin resistance as compared with Group A organisms. The 190-fold increase demonstrated by one organism after 60 transfers and the 107-fold rise observed in another after 80 subcultures[7] indicate that great penicillin resistance may be induced in Group B streptococci. We have not yet attained the upper limit of resistance for any of the strains tested.

The greater ease in acquiring penicillin resistance in Group B than in Group A streptococci is probably of clinical importance. An example of this phenomenon was seen recently in a patient who died of subacute bacterial endocarditis at the Albert Merritt Billings Hospital of the University of Chicago. Shortly before death a strain of Group B streptococci was obtained from his pharynx, and at autopsy from his blood. He had received up to 5 million units of penicillin a day for the previous 30 days.

The loss of mouse virulence, changes in hemolytic characteristics, variations in the colonial appearance, and disappearance of group specificity in some strains after serial transfers on penicillin medium remain unexplained.

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[6] Lancefield, R. C., *J. Exp. Med.*, 1934, **59**, 459.

[7] Unpublished data.

*Summary.* 1. Ten strains of Group B streptococci were transferred serially on media containing increasing quantities of penicillin in an effort to produce resistance. All 10 organisms showed a significant increase in resistance varying from 11 to 190-fold over the parent strains.

2. No increased resistance was induced by serial transfers on control medium.

3. In contrast to Group A organisms, Group B streptococci did not lose resistance on subculture on blood agar or on serial passage through mice.

4. Virulence decreased in all resistant strains of streptococci. The pathogenicity was partially restored in one organism but remained unchanged in two after passage in normal mice.

5. In only 2 resistant strains was the group specificity lost.

6. Transient alterations in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were demonstrated by all strains when grown on maximal concentrations of penicillin. These colonies reverted to the parent type when subcultured on blood agar.

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## 16257

## Antibiotic Studies on Beta Hemolytic Streptococci: III. Penicillin Resistance Acquired by Group C Organisms.

HORACE M. GEZON. (Introduced by C. Phillip Miller.)
*From the Department of Pediatrics, University of Chicago.*

Streptococci of Lancefield Group A or B present group differences as well as strain variations within a group, when transferred serially on media containing penicillin. These characteristics are described in the preceding reports.[1,2] In general, Group A differ from Group B streptococci in having a lower initial penicillin sensitivity. They show less tendency to acquire penicillin resistance *in vitro,* lose acquired resistance more rapidly on passage in mice or plain medium, and develop colonial and hemolytic changes less quickly when growing on penicillin medium.

The initial penicillin sensitivity of Group C organisms is reported[3-5] to vary from 0.0078 to 0.0313 units of penicillin per ml of medium. We have studied 24 recently isolated strains by the Fleming[6] ditch-plate method and have found their sensitivity range to be 0.007 to 0.03 units of penicillin per ml of medium.

The present study was undertaken to determine the similarities and differences between streptococci of Groups A, B, and C as regards their ability to acquire penicillin resistance.

*Results. Acquired Resistance on Medium Containing Penicillin.* Five strains of Group C streptococci underwent serial transfer on blood agar containing penicillin. Three of the 5 organisms after 60 subcultures demonstrated a moderate increase in penicillin resistance varying from 11- to 16-fold. Two forms, $U_3$ and $R_3$, after 60 and 56 transfers respectively, developed only a slightly increased resistance. The results are given in Table I.

*Acquired Resistance on Control Medium.* Four strains of streptococci subcultured from

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[1] Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 208.

[2] Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 212.

[3] Watson, R. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 65.

[4] Simmons, R. T., and Christie, R., *M. J. Australia,* 1946, **1**, 349.

[5] Rutherford, R. H., Marquardt, G. A., and Van Ravenswaay, A. C., *J. Missouri State Med. Assn.,* 1946, 535.

[6] Fleming, A., *Proc. Roy. Soc. Med.,* 1941, **34**, 342.

TABLE I.
Induced Penicillin Resistance in Group C Beta Hemolytic Streptococci.

| Strain | Number of passages | Initial penicillin sensitivity,* u/ml | Final penicillin sensitivity,* u/ml | Fold change |
|---|---|---|---|---|
| $U_1$ | 60 | .01 | .16 | 16 |
| $U_2$ | 60 | .01 | .14 | 14 |
| $U_3$ | 60 | .03 | .14 | 4 |
| $U_4$ | 60 | .02 | .33 | 16 |
| $R_3$ | 56 | $<.01$ | .02 | $> 2$ |

* Penicillin sensitivity is defined as the highest concentration of penicillin in the medium which permits colonies to grow.

TABLE II.
Comparison of the Rate of Development of Penicillin Resistance of Parent and Control Strains of Streptococci.

| Strain | Mean of first 15 transfers of parent* strain on penicillin medium u/ml | Mean of first 15 transfers of control† strain on penicillin medium u/ml |
|---|---|---|
| $U_1$ | .033 | .020 |
| $U_2$ | .030 | .020 |
| $U_3$ | .033 | .016 |
| $U_4$ | .033 | .034 |

* Parent strain is a freshly isolated organism.
† Control strain is the parent form after 60 transfers on control medium.

the original parent organisms were passed on control (*i.e.*, penicillin-free) blood agar. After 60 transfers these bacteria were retested for penicillin sensitivity by the Fleming ditch-plate method. For purposes of comparison alternate lines on plates containing varying concentrations of penicillin were inoculated with the paired resistant and control organisms. No increased penicillin resistance was demonstrated by these control organisms.

In an effort to discover whether adaptation to growth on artificial medium would influence the development of penicillin resistance, the control organisms were carried on penicillin medium for the 61st to 75th passages. The mean values for the first 15 transfers of the parent and the control strains on penicillin medium are given in Table II. Again as was found with both Group A and B streptococci, the repeated subcultivation on plain blood agar does not facilitate the acquisition of resistance to penicillin.

*Loss of Acquired Resistance.* Only one strain, $R_3$, was grown serially on penicillin-free medium and two strains, $U_1$ and $R_3$, passed in untreated mice in an effort to restore the penicillin sensitivity. After 25 transfers on plain blood agar and 12 passages intracerebrally in mice no decrease in the acquired resistance of the organism had occurred.

D. *Change in Virulence.* Using the method described in the first report of this series,[1] mouse virulence studies were performed on all 5 strains. The results appear in Table III. As was observed previously with Group A and B streptococci, all organisms suffered a marked loss of mouse virulence after repeated subcultivation on penicillin-containing medium and relatively little change after a like number of transfers on control medium.

Two resistant strains were passed 12 times

TABLE III.
Mouse Virulence of Group C Streptococci with Acquired Penicillin Resistance.

| Strain | Initial $LD_{50}$* | $LD_{50}$* after 60 transfers on control medium | $LD_{50}$* after 60 transfers on penicillin medium | $LD_{50}$* of resistant strain after 12 mouse passages |
|---|---|---|---|---|
| $U_1$ | .00015 ml | .000096 ml | $>.03$ ml | .006 ml |
| $U_2$ | .0018 | .0015 | $>.03$ | |
| $U_3$ | .0000048 | .000030 | $>.03$ | |
| $U_4$ | .000060 | .00048 | $>.03$ | |
| $R_3$ | .000096 | | $>.03$ | .00096 |

* $LD_{50}$ is stated as the amount of undiluted bacterial suspension in normal saline that will kill 50% of the mice injected. This suspension has a density equal to McFarland No. 1 standard and contains approximately $2 \times 10^9$ organisms per ml.

TABLE IV.
Group Specificity of Streptococci After 56 to 60 Serial Transfers on Penicillin and Penicillin-Free Media.

| | Strain | | | | |
|---|---|---|---|---|---|
| | $U_1$ | $U_2$ | $U_3$ | $U_4$ | $R_3$ |
| Parent culture | C | C | C | C | C |
| Final control culture | C | C | C | C | — |
| Final penicillin resistant culture | C | N | N | N | C |

C = group C.
N = not group A, B, or C.
— = grouping not performed.

in mice in trying to restore the virulence. The $LD_{50}$ values are given in Table III. In both instances only a partial restoration was accomplished.

*Antigenic and Hemolytic Changes.* The results of the serological grouping of the streptococci before and after repeated transfers on penicillin and control media are given in Table IV. In the 4 control organisms studied no loss of the group specific antigen

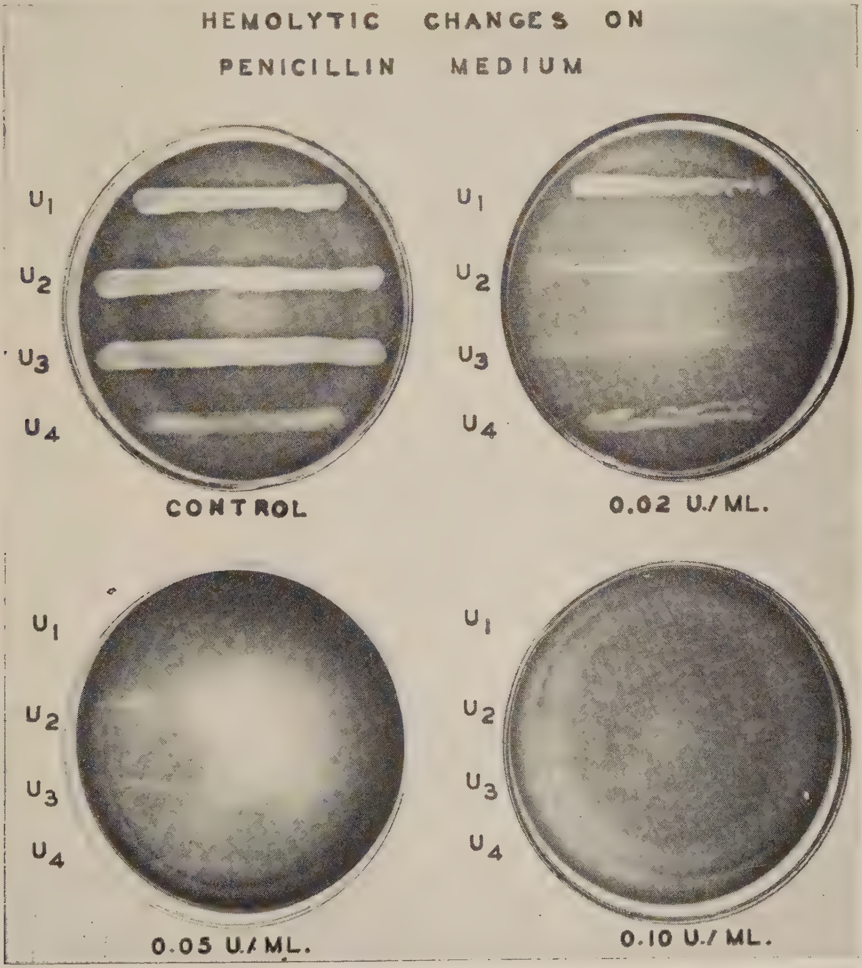

FIG. 1.

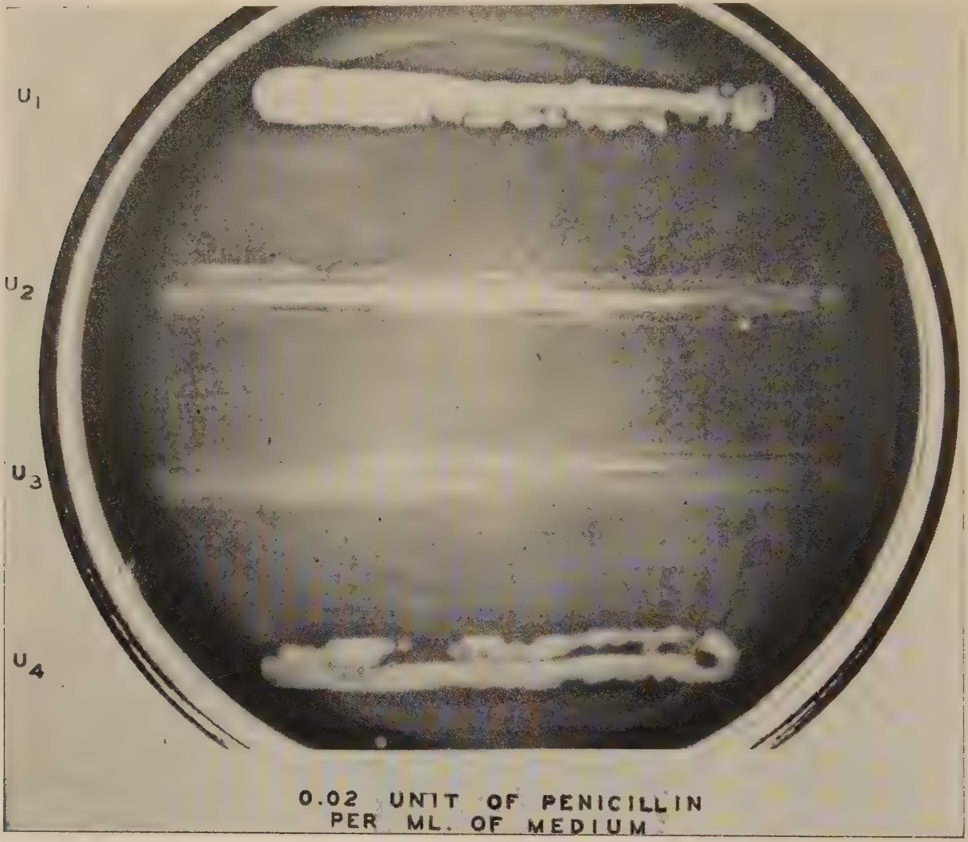

FIG. 2.

occurred. After acquiring penicillin resistance, only 2 of 5 strains were classifiable by the Lancefield technic.

The change in the character of the hemolysis described for Group A and B organisms appeared as well in all 5 strains of Group C streptococci when the organisms were grown on medium containing penicillin. An example of this hemolysis is given in Fig. 1. A close-up view of the 0.02 unit per ml plate of Fig. 1 is shown in Fig. 2. Here the hemolytic changes are best demonstrated by the $U_2$ strain with both green and clear hemolysis present in the same growth. Frequently the transformation to alpha or gamma types of colonies was present in the maximum concentration of penicillin and also at levels below that which would permit growth. Mixtures of alpha, beta, and gamma hemolytic colonies were seen on plates containing a single concentration of penicillin. Some strains of Group C streptococci normally show a zone of greenish hemolysis outside the wide clear zone. Forms with this characteristic display an exaggeration of this color when grown on minimal concentrations of penicillin.

*Discussion and Conclusions.* The behavior of Group C streptococci on penicillin medium resembles in many respects that of Group A and B organisms.[1,2] The same drop in mouse virulence, transient hemolytic changes, and colonial changes were demonstrated by nearly all strains of the 3 groups. In general, Group B streptococci develop a much higher degree of *in vitro* resistance at a faster rate than either Group A or C organisms. The acquired resistance appears to be a more permanent characteristic of Group B and C organisms than of Group A. Only in the latter did passage in normal mice or on penicillin-free medium restore the sensitivity.

If these *in vitro* studies could lead to the conclusion that resistance to penicillin in Group A and C streptococci is not likely to develop, it would be of great clinical importance. However, it should be emphasized that these observations on the group and strain behavior of artificially resistant streptococci, while interesting and valid, must not be assumed to apply by analogy to natural resistance.

*Summary.* 1. Five strains of Group C streptococci were subcultured serially on medium containing penicillin in an effort to induce resistance. Three of the 5 strains developed a 14- to 16-fold increase after 60 transfers. Two strains demonstrated only a 2- to 4-fold increase after similar subcultures.

2. No increased resistance was induced by serial transfers on control medium.

3. Acquired resistant organisms maintained their resistance on serial subcultures on blood agar medium or on serial passages through mice.

4. Decreased mouse virulence was shown by all resistant strains of streptococci. Relatively little change appeared in the control organisms. Lost virulence was partially restored in 2 strains by passage in normal mice.

5. The group specific precipitinogen was demonstrable in only 2 of the 5 resistant strains, but was present in 4 control organisms.

6. Transient changes in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were demonstrated by all strains when grown on maximal concentrations of penicillin. These colonies reverted to the parent type when subcultured on blood agar.

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## 16258 P

# Propagation of the Mammary Tumor Milk Agent in Tumors from $C_{57}$ Black Mice.*

JOHN J. BITTNER.

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If females of the $C_{57}$ black strain obtained the mammary tumor milk agent by nursing females of cancerous strains, mice of some sublines may, when they are maintained as breeders, show incidences of mammary cancer in excess of 50%[1-4] although mice of this strain are generally regarded to have a "low susceptibility" for spontaneous mammary cancer. In 2 studies it was determined that the incidence may be approximately as high,[4] or even higher,[2] in the progeny than in the mice of the fostered generation, demonstrating that the females may propagate and transmit the milk agent. Fostered females and their descendants of a line of the $C_{57}$ black strain which gave a low incidence of spontaneous tumors have also been found to transmit the milk agent.[5]

The milk agent, one of the 3 causative factors for mammary cancer in mice,[6] may be

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* Assisted by grants from the Citizens Aid Society of Minneapolis, the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, the Cancer Research Fund of the University of Minnesota Graduate School, the Minnesota Cancer Society, and the Elsa U. Pardee Foundation.

[1] Bagg, H. J., *International Cancer Research Foundation, Report of Activities During 1939*, p. 14.

[2] Fekete, E., and Little, C. C., *Cancer Research*, 1942, **2**, 525.

[3] Andervont, H. B., *J. Nat. Cancer Inst.*, 1943, **3**, 359.

[4] Haagensen, C. D., and Randall, H. T., *Cancer Research*, 1945, **5**, 352.

[5] Bittner, J. J., *J. Nat. Cancer Inst.*, 1940, **1**, 155.

[6] Bittner, J. J., *Public Health Rep.*, 1939, **54**, 1590.

TABLE I.
Activity of the Milk Agent in Spontaneous Mammary Tumors from C57 Blk. Mice, Line 6.

| Tumor No. | Date injected | Gm.Eq. inj. | No. | % Ca | % living |
|---|---|---|---|---|---|
| 7476 ($A_2$)* | 6/27/46 | 1/50 | 19 | 79 | 16 |
| | | 1/1000 | 13 | 46 | 21 |
| 7477 ($A_2$)* | ” | 1/50 | 29 | 55 | 14 |
| | | 1/1000 | 25 | 60 | 36 |
| 7478 ($Z_1$) | ” | 1/100 | 31 | 58 | 23 |
| 7788 ($Z_1$) | 3/26/47 | 1/50 | 56 | 30 | 67 |
| | | 1/1000 | 32 | 25 | 69 |

* Tumors inoculated.

TABLE II.
Comparative Activity of the Milk Agent in Tumors from C57 Blk. Mice.

| | | Tumor No. 7476 | | | Tumor No. 7477 | | |
|---|---|---|---|---|---|---|---|
| Tissue extracted | Gm.Eq.inj. | No. | % Ca | % liv. | No. | % Ca | % liv. |
| Spontaneous tumors | 1/50 | 19 | 79 | 16 | 29 | 55 | 14 |
| | 1/1000 | 13 | 46 | 21 | 25 | 60 | 36 |
| Transplants: after 1 passage | 1/50 | 9 | 67 | 22 | 29* | 100 | — |
| | 1/1000 | 21 | 48 | 29 | 20 | 65 | 25 |
| Transplants: after 10 passages | 1/10 | 38 | 39 | 58 | 38 | 66 | 34 |
| | 1/1000 | 44 | 25 | 73 | 44 | 16 | 82 |

* Average cancer age, 371 days.

recovered from extracts of spontaneous mammary cancer[7-9] from mice of cancerous strains and has been found to survive for at least 10 passages of these tumors in mice which did not themselves have the milk agent.[10]

In this study we have investigated the presence of the milk agent in 4 spontaneous mammary tumors which developed in mice of Line 6 of the $C_{57}$ black stock. Two tumors, Nos. 7476 and 7477, arose in the progeny of a female which had obtained the milk agent from a foster mother of the cancerous A strain. The mother of the $C_{57}$ black mice died cancerous. The other tumors, Nos. 7478 and 7788, developed in females which had been nursed by a female of the cancerous $C_3H$ stock. In addition, tumors 7476 and 7477 were transplanted into mice of the $C_{57}$ black stock which had not received the milk agent, either from their mother or a foster mother. The transplanted tumors were tested after the first passage and also after the tenth passage for the survival of the milk agent.

The test animals were ZBC mice derived by reciprocal matings between mice of the fostered A and $C_3H$ strains. Controls have been found to have few spontaneous mammary tumors whereas when the ZBC mice obtain the milk agent, high incidences have been observed.[11]

The tumor extracts were all prepared in the same manner. The tissue was ground in a mortar with sand and suspended in either physiological saline or triple distilled water (1:10 by weight). The suspension was centrifuged for 7 to 10 minutes at approximately 2,500 r.p.m. and 1 cc of the supernatant, at various dilutions, was injected intraperitoneally into the test animals. The injected mice were continued as breeders to insure an adequate hormonal stimulation for the genesis

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[7] Bittner, J. J., *Science*, 1941, **93**, 527.

[8] Barnum, C. P., Ball, Z. B., Bittner, J. J., and Visscher, M. B., *Science*, 1944, **100**, 575.

[9] Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **5**, 143.

[10] Bittner, J. J., Evans, C. A., and Green, R. G., *Science*, 1945, **101**, 95.

[11] Bittner, J. J., *AAAS, Research Conference on Cancer, 1945*, p. 63.

of mammary cancer.

The data are presented in Tables I and II. The results demonstrate that the mammary tumor milk agent may be recovered from spontaneous mammary cancers which developed in mice of the $C_{57}$ black stock and may be propagated in the transplants of these tumors. Two of the tumors arose in mice which had received the agent from their mothers, again demonstrating that females of the $C_{57}$ black strain, at least some sublines, may transmit the agent in the milk.

*Summary.* Mice of some sublines of the $C_{57}$ black stock may propagate and transmit the mammary tumor agent. The agent may be recovered from spontaneous mammary cancers which developed in mice of the strain and may survive for at least 10 passages of these tumors in mice without the agent.

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## 16259

# Effect of Bacterial Endotoxins on Carbohydrate Metabolism of Rabbits.*

Ernest Kun and C. Phillip Miller.†

*From the Departments of Pharmacology and Medicine, University of Chicago.*

The injection into animals of heat-killed cultures of certain bacterial cells has been shown to produce marked hyperglycemia.[1,2] Diphtheria toxin which has been studied more extensively[3] causes a similar rise in blood sugar and depletion of tissue carbohydrate. Some investigators[4,5] have concluded that diphtheria toxin affects metabolism by damaging the liver, causing increased glycolysis and diminished phosphorolysis. The biochemical mechanism by which the metabolites or cellular constituents of bacteria interfere with the metabolism of the infected animal is not well understood.

The experiments described below concern the effects of meningococcal and Salmonella endotoxins on the intermediate carbohydrate metabolism of rabbits. As in experiments already reported,[6] the experimental conditions may be defined as acute endotoxin poisoning. The effect of a lethal dose of endotoxin was studied during the short period of intoxication which terminated in the death of the animal in 1.5-3 hours. It is believed that, under such circumstances, the effect of the toxin is not significantly altered by spontaneous changes in metabolism.

*Materials and Methods.* The endotoxins were sterile, unpurified preparations made from washed bacterial cells by the method already described.[6] The intravenous dose which was lethal in 2 to 3 hours was 20-50 mg per kg. Rabbits weighing 1.5-3 kg were used. The experimental and control animals were fasted for 24 hours before each experiment. No anesthesia was used. Blood samples were taken by cardiac puncture. Tissue analyses were carried out immediately after the death of the animal. Pairs of rabbits having almost identical weights were kept under the same environmental conditions throughout each experiment. One rabbit in each pair was injected with endotoxin and the other kept as a control and sacrificed when death occurred in the former. In some

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* This investigation was undertaken and supported jointly by the U. S. Navy, Office of Naval Research, and the University of Chicago.

† With the technical assistance of Mrs. S. E. Gurnee.

[1] Menton, M. L., and Manning, H. M., *J. Med. Res.*, 1924, **44**, 675.

[2] Zweckwer, L. T., and Goodell, H., *J. Exp. Med.*, 1925a, **42**, 43; *J. Exp. Med.*, 1925b, **42**, 57.

[3] Holmes, E., *Physiol. Rev.*, 1939, **19**, 439.

[4] Soskin, S., Allweiss, M. D., and Mirsky, J. A., *Arch. Int. Med.*, 1935, **56**, 927.

[5] Taubenhaus, M., and Soskin, S., *J. Clin. Endocrin.*, 1942, **2**, 171.

[6] Kun, E., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 197.

instances the animals were sacrificed before death and the same changes were found in the concentration of metabolites and enzyme activities as in the animals which succumbed to endotoxin.

Blood glucose was determined by the method of Nelson,[7] lactic acid according to Barker and Summerson,[8] pyruvic acid as described by Elgart and Nelson,[9] and acid soluble phosphorus by the method of Schneider.[10] Glycogen was determined as glucose.[11]

*Results.* The results of the analyses for blood sugar, lactic acid, phosphorus and pyruvic acid are shown in Fig. 1, 2, 3, and 4. The graphs were constructed from data obtained from experiments of identical length. Each point represents the blood values of one individual animal.

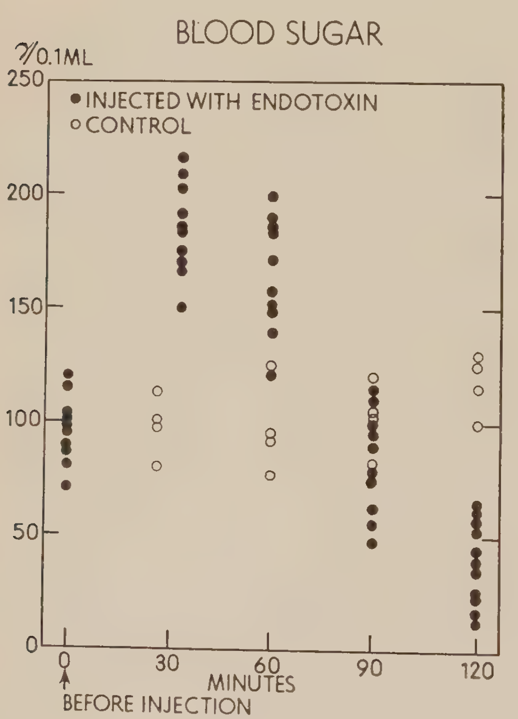

FIG. 1.

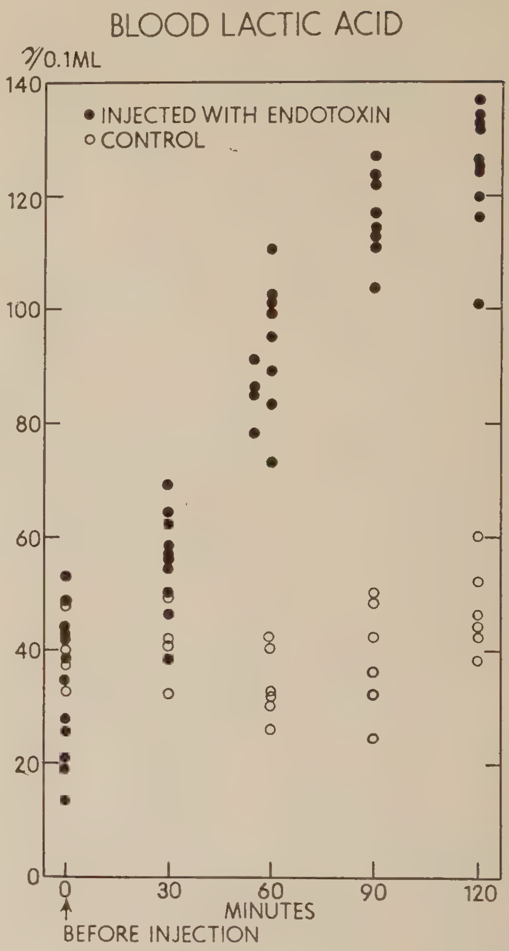

FIG. 2.

The results show that the endotoxins caused hyperglycemia which reached its maximum 30 minutes after the injection and was followed by hypoglycemia. Blood lactic acid showed marked increase. Blood phosphorus fell slightly a few minutes after the injection and then rose sharply. The blood pyruvic acid content decreased after the injection of the toxin and remained below the normal level.

The results of the tissue analyses are given in Fig. 5, in which the liver and muscle glycogen, lactic acid and pyruvic acid of normal animals are compared with the results obtained from animals injected with meningococcal endotoxin. (Eight pairs of rabbits were used for glycogen, 5 pairs for tissue

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[7] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

[8] Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

[9] Elgart, J. S., and Nelson, N., *J. Biol. Chem.*, 1941, **138**, 443.

[10] Schneider, W., *J. Biol. Chem.*, 1945, **161**, 293.

[11] Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, Burgess Publishing Company, 1945.

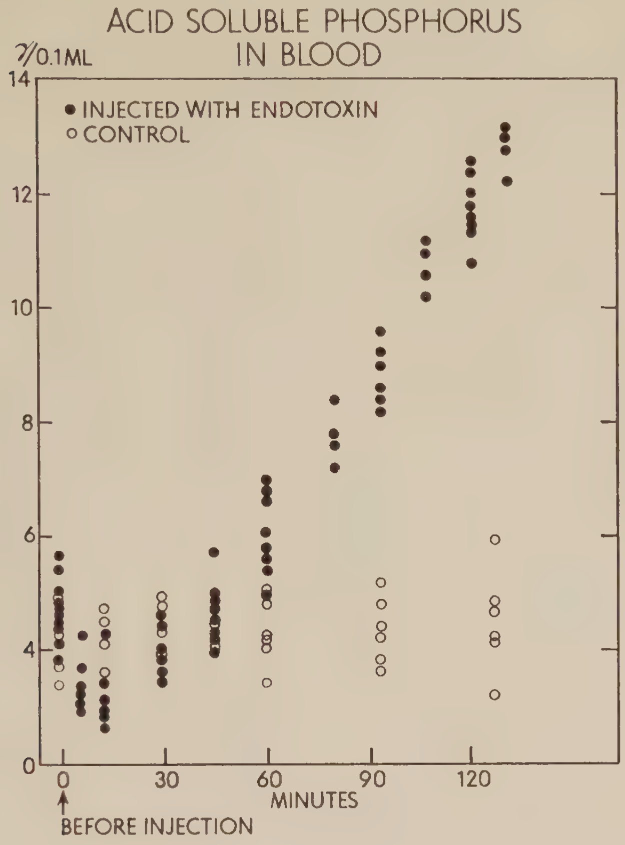

FIG. 3.

lactic acid and 4 pairs for tissue pyruvic acid determinations.)

The greatest difference between the control rabbits and those injected with endotoxin was found in the glycogen content of liver and muscle, particularly in the former, as the livers of the animals poisoned by endotoxin contained only traces of glycogen.

The lactic acid content of the liver and muscle in the endotoxin treated rabbits was markedly increased, whereas the pyruvic acid content was diminished.

It is well established that in mammalian tissue under normal circumstances glycogen is broken down to pyruvate which is further oxidized through various channels. If the aerobic conditions in tissues are altered in some way, accumulation of lactate and inorganic phosphate occurs.[12] Our observations on these constituents in the blood and tissues of our experimental animals suggested, therefore, that the injection of the bacterial endo-

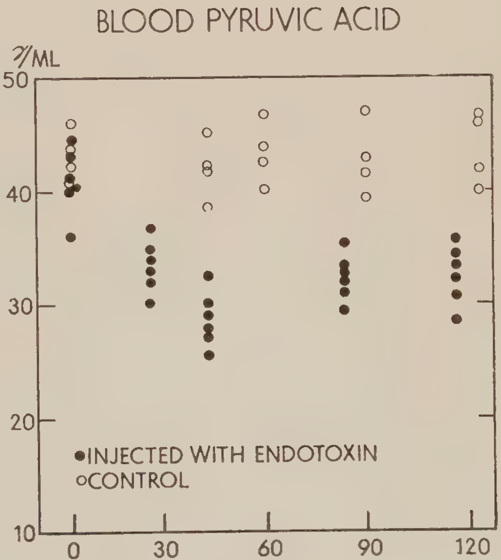

FIG. 4.

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[12] Barron, E. S. G., *Advances in Enzymology*, 1943, **3**, 149.

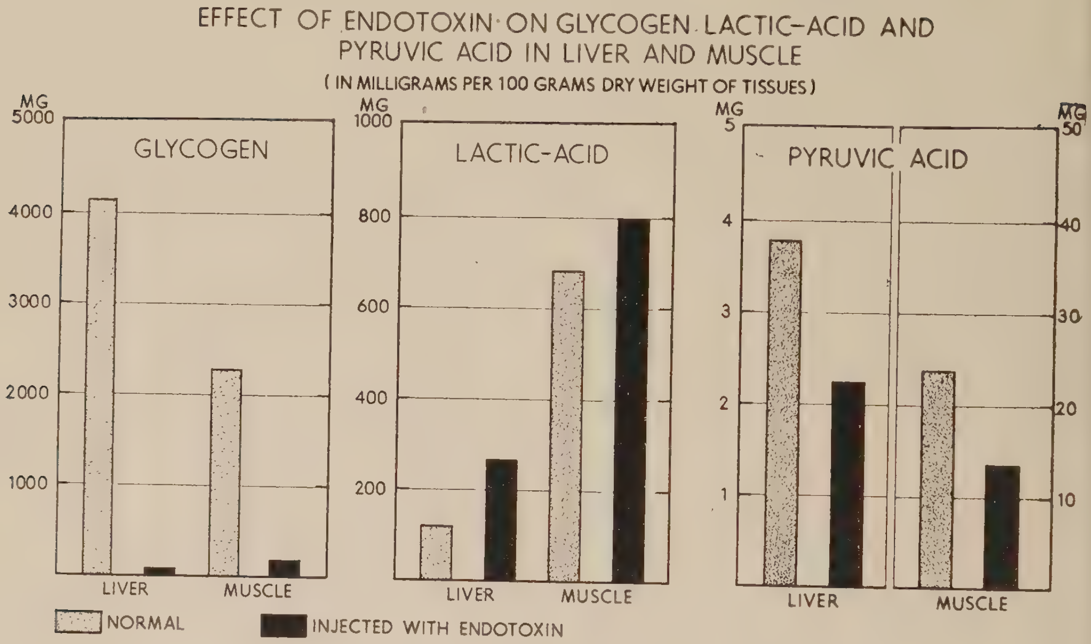
EFFECT OF ENDOTOXIN ON GLYCOGEN LACTIC-ACID AND PYRUVIC ACID IN LIVER AND MUSCLE
(IN MILLIGRAMS PER 100 GRAMS DRY WEIGHT OF TISSUES)

FIG. 5.

TABLE I.
Succinic Dehydrogenase Activity of the Liver and Muscle of Rabbits Injected with Endotoxin.

| | Liver | | Muscle | |
|---|---|---|---|---|
| | Normal control | Endotoxin | Normal control | Endotoxin |
| Meningococcal endotoxin | 88.4 | 28.0 | 16.2 | 6.0 |
| | 60.1* | 40.2* | 15.1* | 6.5* |
| | 68.9 | 29.7 | 24.2 | 8.4 |
| | 80.0 | 16.0 | 26.1 | 8.7 |
| | 65.4* | 20.4* | 19.3* | 7.1* |
| | 72.4* | 31.0* | 29.2* | 6.9* |
| Salmonella endotoxin | 62.4 | 26.1 | 19.6 | 5.2 |
| | 79.7 | 15.1 | 17.1 | 7.1 |
| | 72.4* | 20.0* | 25.3* | 8.0* |
| | 80.1 | 17.8 | 22.0 | 9.2 |

* These values were obtained from animals which were sacrificed before death from the toxin.

toxins might have produced a state of anoxia in their tissues.

In order to learn more about the mechanism of the tissue anoxia caused by endotoxin, rabbits were injected with meningococcal and Salmonella endotoxin‡ as described above and the cytochrome oxidase and succinic-dehydrogenase activity of their muscle and liver were determined by the Warburg technic as described by Schneider and Potter.[13] In these enzyme assays phosphate buffer (pH 7.4) was used instead of $H_2O$ for the preparation of tissue homogenates. The importance of this technical detail will be discussed elsewhere. A significant inhibition of succinic dehydrogenase activity was demonstrable. Cytochrome oxidase was not affected (see Table I).

It is interesting to note that the endotoxins of both Salmonella and meningococcus produced similar effects not only in their inhibition of succinic-dehydrogenase but also on the carbohydrate metabolite concentration of tissues. Since the enzyme inhibition occurred in a few minutes after the injection of the endotoxin, it is reasonable to assume that the inhibition of this enzyme activity plays an important role in the mechanism responsible for the changes in carbohydrate metabolite concentration in the poisoned rabbit tissues. The mechanism of this enzyme inhibition and its effect on carbohydrate metabolism is being investigated in detail.

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‡ The endotoxin of *Salmonella aertrycke* was prepared in the same way as meningococcal endotoxin.

[13] Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, **149**, 217.

*Summary.* The intravenous injection of meningococcal or Salmonella endotoxin into rabbits produced increase in blood glucose, lactic acid and inorganic phosphorus. This was followed by hypoglycemia which could be observed before the death of the animal. Liver and muscle glycogen decreased while lactic acid content of the tissues increased. The pyruvic acid content of blood and tissues showed a significant decrease. Succinic dehydrogenase in both muscle and liver was markedly inhibited. Cytochrome oxidase activity was not affected.

The Na-ascorbate used in the enzyme assays was generously supplied by Van Patten Pharmaceutical Company, Chicago.

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## 16260

# Passive Cellular Transfer of the Tuberculin Type of Hypersensitivity.*

ABRAM B. STAVITSKY.† (Introduced by D. H. Campbell.)

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Chase[1] recently reported that the tuberculin type of hypersensitivity was transferred passively to normal guinea pigs by injection of the cells of peritoneal exudate, lymph nodes or spleen of guinea pigs sensitized to tuberculin by the injection of heat-killed tubercle bacilli. The passively conferred sensitivity was transient, lasting about 5 days. This is a significant achievement as the failure passively to transfer tuberculin sensitivity has been adduced as a basic distinction between the tuberculin and the anaphylactic or Arthus types of hypersensitivity.[2] It was hoped that, if confirmed, the passive cellular transfer of tuberculin hypersensitivity might provide a method suitable for the elucidation of the underlying chemical factors in the induction and development of this peculiar allergic phenomenon. The repetition of Chase's studies was therefore undertaken.§

*Materials and Methods.* The materials and methods were essentially those used by Chase[1] in his experiments. White albino guinea pigs of both sexes were sensitized to tuberculin by subcutaneous injection into the groin of heat-killed human tubercle bacilli suspended in mineral oil[3,4,5] and aquafor. Usually 2.5 mg of dried bacillary growth was suspended in a total inoculum of 1 ml for each animal. Five to 9 weeks later, when cutaneous sensitivity to

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* Supported by a grant from the Rockefeller Foundation for research in immuno-chemistry.

† Present address: Institute of Pathology, Western Reserve University, Cleveland, Ohio.

‡ Contribution No. 1153.

[1] Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 134.

[2] Rich, A. R., *The Pathogenesis of Tuberculosis*, Springfield, Charles C. Thomas, 1944.

§ Since these confirmatory studies have been completed another group of workers reported successful repetition of Chase's experiments. Cummings, M. M., Hoyt, M., and Gottshall, R. Y., *Pub. Health Rep.*, 1947, **62**, 994.

[3] Freund, J., Casals-Ariet, J., and Hosmer, E. P., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 509.

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TABLE I.
Reactions to Tuberculin in Guinea Pigs Injected with Cells from Normal and Tuberculin-sensitized Guinea Pigs.

| No. and type donor animals | Volume and type material transferred to each recipient* | No. of recipients* | Avg reaction (mm) of recipients 24 hr | and 48 hr after injection of tuberculin† | No. tuberculin pos.‡ / No. tested |
|---|---|---|---|---|---|
| 15 sensitized | 0.5 ml peritoneal cells | 3 | 8 × 9, i, e | 12 × 13, i, e | 3/3 |
| 16 " | 0.65 " " " | 4 | 8 × 8, i, e | 10 × 12, i, e | 3/4 |
| 4 " | — " " | 1 | 8 × 8, e | 12 × 15, i, e | 1/1 |
| 2 " | — " " | 1 | 7 × 7, i | 10 × 10, i, e | 1/1 |
| 9 " | 2.0 ml splenic cells | 3 | 10 × 9, i, e | 12 × 12, i, e | 3/3 |
| 9 " | 2.0 " lymph node cells | 3 | 10 × 10, i, e | 12 × 12, i, e | 2/3 |
| 9 " | 19.0 " defibrinated blood | 3 | 9 × 9, i, e | 10 × 11, i, e | 1/3 |
| 9 " | 20.0 " citrated blood | 3 | 8 × 8, i, e | 11 × 11, i, e | 1/3 |
| 12 " | 8.0 " serum | 4 | 0 | 0 | 0/4 |
| 16 normal | 0 70 " peritoneal cells | 4 | 0 | 0 | 0/4 |
| 16 " | 2.25 " splenic cells | 4 | 0 | 0 | 0/4 |

* For example, in the first group peritoneal cells from 15 sensitized animals were divided into 3 parts and injected into each of 3 animals; in second group, cells from 16 animals were divided into 4 parts, and so on.

† Reactions to second injection of tuberculin 48 hours after passive transfer. i = induration; e = erythema; 0 = negative.

‡ In first group, for example, 3 out of 3 animals tested reacted positively to tuberculin; in second group 3 out of 4, and so on.

tuberculin was found to be definite and intense, exudates were evoked in the sensitized guinea pigs by the intraperitoneal inoculation of 30 ml of mineral oil. Forty-eight hours later the peritoneal cavities were flushed out with citrated-Locke's solution containing 0.25% gelatin. The washings were placed in a separatory funnel and allowed to stand. After the oil had risen the aqueous layer was collected and centrifuged lightly for several minutes. The sedimented cells, about 0.15 ml from each animal, were resuspended in citrated gelatin-Locke's solution and again centrifuged. The washed cells were suspended in about 4 ml of gelatin-Locke's solution and immediately inoculated intraperitoneally into male albino guinea pigs. The differential cell count of the suspension before inoculation revealed about 25% polymorphonuclear leukocytes, 35% lymphocytes, and 40% mononuclears.

Twenty-four and 48 hours after the cell-transfer the recipient animals were tested for tuberculin sensitivity by intradermal injection of 1:2 or 1:4 Old Tuberculin from which the glycerin had been removed by precipitation with cold alcohol.[6]

As a control each animal was also skin-tested with 0.1 ml of 1:2 or 1:4 glycerin broth which was deglycerinated in the same manner as was the Old Tuberculin. It had been previously determined that these concentrations of "deglycerinated" tuberculin and broth were the highest not producing visible reactions upon injection into the skin of normal, unsensitized guinea pigs. The cutaneous response to all test inoculations was measured 24 and 48 hours after inoculation of the test material.

Cells from the spleen and lymph nodes and, in a few instances, whole defibrinated or citrated blood and serum from sensitized animals also were employed in the attempt to transfer sensitivity. It is important to note that smears of the lymph node cell suspensions revealed that approximately 95% of the cells were small or medium-sized lymphocytes and 5% monocytes. As controls corresponding types of cells from normal, unsensitized guinea pigs were inoculated into normal guinea pigs.

*Results.* Pertinent data and results of these experiments are summarized in Table I. In order to conserve space the uniformly negative results of control injections of "deglycerinated" broth and of all tests 24 hours after the cell transfer are omitted from the table.

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[6] Chase, M. W., personal communication.

In general the data confirm Chase's results on the passive transfer of tuberculin hypersensitivity with the further observation that whole blood may occasionally transfer sensitivity. In a few instances by means of daily skin tests it was found that the passively conferred sensitivity lasted only 4 or 5 days. The occasional failures in transfer of sensitivity with peritoneal exudate or lymph node cells have not been satisfactorily explained.

*Discussion.* It should be emphasized that the positive tuberculin reactions elicited in the recipient animals were of the delayed type typical of reactions in animals actively sensitized by injection of tubercle bacilli.[2] The reactions rarely appeared much before 24 hours and were at their height of intensity 48 to 72 hours after injection.

It is interesting to note that sensitivity was transferred in 2 instances by injection of whole blood from sensitized animals. This is not surprising as Chase has found recently[6] that the buffy coat separated from the red cells of the blood may passively transfer sensitivity. These observations and the demonstration of typhoid agglutinins in the buffy coat from the blood of immunized rabbits[7] suggest a wider than hitherto supposed participation of the white cells of the blood in immunological phenomena.

The positive results employing suspensions containing about 95% lymphocytes are indicative of the possible preeminence of this cell in the transfer of sensitivity. The specific lysis of a portion of small mature lymphocytes from blood or spleen of tuberculous mice and guinea pigs upon contact with tuberculin[8] may be related to the ability of lymphocytes to transfer sensitivity. The recent implication of lymphocytes as cells which synthesize antibodies[9] may or may not be a related finding, as it has not been shown so far that antibodies are involved in the development of tuberculin hypersensitivity.[2] The demonstration[10] that cells from tuberculin-sensitive animals may retain their sensitivity to the toxic action of tuberculin after several transplants in tissue culture would seem to rule out the role of circulating antibodies in this phenomenon.

Work is proceeding in the attempt to isolate the factor responsible for the transfer of sensitivity. Thus far efforts to transfer sensitivity by means of various crude extracts of the cells have been completely negative. In the endeavor to determine whether the cell transfer is a general phenomenon, cell transfer of the delayed type of pneumococcus sensitivity in rabbits[11] has been tried. Thus far these experiments also have proven unsuccessful.

*Summary.* 1. In confirmation of Chase's studies, the tuberculin type of hypersensitivity has been transferred passively to normal, unsensitized guinea pigs by injection of the cells of peritoneal exudate, lymph nodes, spleen, and whole blood of tuberculin-sensitive guinea pigs. The passively conferred sensitivity lasted 4 or 5 days.

2. The transfer of sensitivity by injection of whole blood taken with other observations suggests that the white cells of the circulating blood may participate more widely than previously believed in immunological phenomena.

3. The positive results employing suspensions containing 95% of lymphocytes suggest that this cell type may be of great importance in the transfer of sensitivity.

The author is grateful to Dr. Dan H. Campbell for interest and encouragement in the course of these studies.

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[7] Stavitsky, A. B., unpublished observations.

[8] Favour, C. B., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 269.

[9] Ehrich, W. E., and Harris, T. N., *Science*, 1945, **101**, 28.

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16261

# 'Accelerator Globulin" and "Antihemophilic Globulin" in Thrombin Formation from *Aged* Prothrombin and in Hemophilic Blood.*

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Recent studies[1-9] have shown that a newly recognized factor is important in the conversion of prothrombin to thrombin. The plasma "accelerator globulin" of Ware, Guest, and Seegers,[3,4,9] used in the present studies, contains this factor and is, in all probability, similar to Quick's "labile factor"[6] and Owren's "factor V."[1] "Antihemophilic globulin"[10] is a plasma protein fraction which restores the "thromboplastic" defect and thus accelerates the thrombin formation in hemophilic blood. The following experimental data were obtained during current *in vitro* studies of the various factors involved in the process of thrombin formation from purified prothrombin preparations.

*Reagents* (*cf.* [11,12]). 1. *Borate buffer* (buff.), pH 7.7, solvent and diluent throughout. 2. *Prothrombin* (PRO.) from bovine plasma, courtesy of Dr. W. H. Seegers and colleagues (Wayne Univ.) PRO. *G* partially purified (product 5 of reported procedures)[13] potency 720 "units" per mg (dry wt). PRO. *H*, highly purified (ammonium sulfate fractionation of product 8),[13] potency 990 "units" per mg (dry wt = 10 × tyrosine). 3. *Thromboplastin* (tpln.) 0.2% rabbit lung preparation (Squibb's). 4. $CaCl_2$ (Ca) M/20. 5. *Fibrinogen* (B.F.), 0.5% Armour's bovine plasma Fraction I (55% "clottable"). 6. *"Antihemophilic globulin"* (HF), Harvard[14] human Fraction I, low in fibrinogen ($HF_1$ 37%; $HF_2$ 18%). 7. *"Accelerator globulin"* (AcG), from bovine plasma (Seegers *et al.*), contained a small amount of prothrombin.

*Methods.* 0.1% solutions of PRO., AcG, and HF were divided into 2 portions, part being freshly frozen and kept at –20°C until tested and part being aged at 28°C (thermostat water bath) for 7 days. 5 cc (total vol.) of thrombic mixtures (T), in borate buffer, contained 0.1% PRO., to dilution noted in Tables I, II, 0.5 cc Ca, 0.5 cc tpln (approximately optimal), and 0.5 cc of the various agents noted. At stated incubation periods (i.t.), 0.25 cc T was added to 0.5 cc BF, at 28°C, and clotting-time (c.t.) noted.

"Antihemophilic" activity was measured by addition of 2 cc of fresh hemophilic blood to 0.1 cc of test preparations and measurement of the coagulation time at 37°C.

*Results.* In the data of Table I, the prothrombin (PRO *H*) after *aging* 1 week at 28°C, when diluted and activated with the stated amounts of calcium and thrombo-

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* This is the sixth of a series of studies aided by a grant from the John and Mary R. Markle Foundation.

† Postdoctorate Fellow, U. S. Public Health Service.

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[5] Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

[6] Quick, A. J., *Am. J. Physiol.*, 1947, **151**, 63.

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[12] Ferguson, J. H., Travis, B. L., and Gerheim, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 285, 302.

[13] Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., *Arch. Biochem.*, 1945, **6**, 85.

[14] Edsall, J. T., *Adv. Prot. Chem.*, 1943, **3**, 383.

TABLE I.
Effects of "Accelerator Globulin" (AcG) and "Antihemophilic Globulin" (HF) on Activation of *Aged* and *Frozen* Prothrombin (Pro.).

T = thrombic mixtures, containing (final concentrations) Pro.*H* (1:20,000), tpln. (1:5000), Ca (M/200), and agents cited (1:10,000), with borate buffer (pH = 7.7) to 5 cc vol. Clotting times (c.t.), sec., at 28°C, for 0.25 cc T + 0.5 cc BF (0.5%), at stated incubation periods (i.t.).

| T. | Pro. | Agent | Incubation time | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 5m | 10m | 15m | 20m | 30m | 1 hr | 4 hr |
| | | | Clotting time (sec.) | | | | | | |
| 1. | Aged | — | 190 | 129 | 105 | 72 | 50 | 30 | 17 |
| 2. | ” | AcG (aged) | 137 | 104 | 78 | 61 | 43 | 23 | 15 |
| 3. | ” | ” (froz.) | 17 | 11 | 10 | 10 | 9½ | 8 | 8 |
| 4. | ” | HF$_1$ (aged) | 165 | 107 | 101 | 98 | 75 | 47 | 30 |
| 5. | ” | ” (froz.) | 161 | 122 | 106 | 100 | 91 | 58 | 32 |
| 6. | Froz. | — | 16 | 10 | 8½ | 8 | 8 | 8 | 8 |
| 7. | ” | AcG (aged) | 12 | 9½ | 8 | 8 | 8 | 8 | 8 |
| 8. | ” | ” (froz.) | 12 | 8 | 8 | 8 | 8 | 8 | 8 |
| 9. | ” | HF$_1$ (aged) | 26 | 16½ | 13 | 12½ | 12 | 12 | 12 |
| 10. | ” | ” (froz.) | 29 | 19 | 15 | 13 | 13 | 12½ | 12½ |
| 11. | — | AcG (froz.) | 120 | 95 | 73 | 71 | 62 | 53 | 48 |
| 12. | — | ” (aged) | 220 | 220 | 225 | 220 | 240 | 300 | 345 |

plastin, showed very slow activation ($T_1$) which was incomplete even after 4 hours incubation. *Aged* AcG exerted only a negligible improvement ($T_2$) as compared with *frozen* AcG ($T_3$) which was able, in less than an hour, to restore the full thrombic potency as shown by the 8″ c.t. identical with that reached in $T_6$, $T_7$, $T_8$. The prothrombin kept in the *frozen* state, when activated in the usual manner with Ca and tpln. ($T_6$) completed the thrombin formation in less than 20 min., and showed only slight acceleration by *aged* AcG ($T_7$) and still minor improvement (10 min. i.t.) by *frozen* AcG ($T_8$). HF$_1$ did not show any accelerator effects with either prothrombin, but, on the contrary, evinced some definite interference with thrombin formation, though somewhat less with *aged* HF ($T_4$, $T_9$) than with *frozen* HF ($T_5$, $T_{10}$). Small amounts of fine flocculant fibrin which appeared during the incubation of these mixtures did not interfere with the tests and were not considered significant.

*Controls.* The fibrinogen (BF) contained a mere trace of prothrombin which caused negligible fibrin shreds to appear in about 25 minutes when tested with the same amounts of Ca and tpln. (in buff.) as used in the other tests. HF controls, similarly activated, barely increased these traces of fibrin, now appearing in about 20 minutes. The AcG preparation, as expected, did show a little content of prothrombin estimated at less than 10%, in the *frozen* solution ($T_{11}$). The *aged* AcG showed no prothrombin activity but did contain a trace of active thrombin which deteriorated somewhat as the incubation proceeded ($T_{12}$). It is not conceivable that these very minor sources of prothrombin and thrombin could be at all significant in explaining the "accelerator" effect noted in $T_3$.

Similar experiments with a less purified prothrombin (PRO. *G*) gave exactly the same results. In Table II, A the data are presented as "percentages" of thrombin yield, derived from the clotting-times (Table II, B) obtained by a series of dilutions of $T_2$ at maximal activity (4 hrs). $T_1$: the *aged* PRO., diluted and activated in the usual manner with optimal Ca and tpln., is only about one-third activated in 4 hrs but is estimated as 80% complete in 3 days. $T_2$: the addition of 1 cc *fresh* (frozen) 1:1000 AcG gave over 90% of maximal thrombin formation in 10 min. and 100% (complete activation) in less than 4 hours. $T_3$: HF$_2$, another "antihemophilic globulin" preparation, one-tenth vol. (0.5 cc) of *fresh* 1:1000 soln., showed the same kind of retarding action previously noted with HF$_1$ (Table I), the best value for thrombin yield (i.t. 2-3 days) not exceeding 20%.

Although PRO. *G* was elsewhere[11] shown

TABLE II.

A. Thrombin Yield, ‘‘percentage’’ activation, after varying periods of incubation (i.t.), in mixtures (T) containing PRO.*G.* (1:12,500) aged 7 days at 28°C, tpln. (1:5000), Ca (M/200), and added agents (mg per cc) noted. 0.25 cc T + 0.5 cc BF. 28°C. pH = 7.7.

| T. | Agent | 5 min | 10 min | 1 hr | 4 hr | 1d | 2d | 3d (i.t.) | Clot lysis (37°) |
|---|---|---|---|---|---|---|---|---|---|
| 1. | buff. | tr. | tr. | 17 | 34 | 50 | 75 | 80 | None (5 days) |
| 2. | AcG (0.2) | 44 | 93 | 95 | 100 | 100 | 96 | 80 | ’’ ’’ |
| 3. | $HF_2$ (0.1) | tr. | tr. | 3 | 6 | 15 | 20 | 20 | ’’ ’’ |

B. ‘‘Percentage’’ Dilutions of $T_2$ (4 hr incub. time): clotting-times (sec.).

| Dil. | 100% | 80% | 60% | 40% | 20% | 10% | 5% |
|---|---|---|---|---|---|---|---|
| Coag. time (sec) | 10 | 15 | 18 | 21½ | 37 | 46½ | 89 |

TABLE III.

‘‘Antihemophilic’’ Activity.

2 cc freshly drawn hemophilic blood added to 0.1 cc 0.1% solutions of agents cited. Coagulation times (C.T.), min., at 37°C.

| Agent: | Saline (control) | AcG (froz.) | AcG (aged) | HF (froz.) | HF (aged) |
|---|---|---|---|---|---|
| Coag. time (min.) | 142 | 98 | 98 | 27 | 60 |

to contain a trace of protease contaminant, the clot-lysis tests recorded in Table II, A show no appreciable enzyme activity in the dilution used.

In other (not cited) experiments with PRO. *G* solution freshly prepared and activated (a) with and (b) without fresh (frozen) AcG, a maximal thrombic potency represented by c.t. of 8 sec. was reached in (a) 20 min. or (b) 30 min., respectively. This confirms the evidence in Table I ($T_5$, $T_7$) showing that these *fresh* or frozen prothrombin preparations contain an abundance of the AcG factor. In earlier experiments,[11] testing stronger (0.2-0.4%) solutions of this same prothrombin, identical activation data (with optimal Ca and tpln.) were obtained with fresh solutions as compared with the same solutions after 3-4 weeks storage at ice-box temperature (5°C).

Table III shows the antihemophilic activity of AcG and $HF_1$. Even in the high (<0.005% *final*) dilution, frozen $HF_1$ showed marked acceleration of clotting. This activity was greatly diminished by aging. AcG both frozen and aged showed only minimal effects.

*Discussion.* The data clearly show that the major change in "aged" prothrombin is an impaired ability to form thrombin with the classical activators, namely, calcium and thromboplastin. Fresh (or frozen) "accelerator globulin" greatly speeds up the conversion and restores the thrombin yield (*cf.* [7]), in fact completely in $T_3$ (Table I), but "aging" of the AcG causes it to lose this property. Fresh (or frozen) prothrombin evidently contains an adequacy of this factor, as a contaminant [6] but it deteriorates on aging at or above room temperature. This deterioration is emphasized by dilution of the prothrombin preparation. There is little doubt that the validity of estimations of the thrombin-yielding potency of prothrombin depends upon adequate control of the "accelerator" factor.[8] Our findings in regard to highly purified (Seegers) prothrombin are comparable to the data of other workers investigating the thrombin-yield changes in stored and otherwise treated plasma. Interpretation in terms of presence or instability of AcG possibly explains most of the divergence of viewpoints noted in the literature and accounts for the prothrombin preserving and activity restoring powers credited to plasma[15] or certain fractions,[1,5,15] including some fibrinogens.[7,8,16]

Questions of possible deterioration of prothrombin proper or of thrombin in highly dilute solutions[11] are raised by the later data of Table II, but the analysis of these problems is beyond the scope of the present inquiry. The same is true of the explanation for the evident interference in thrombin formation by our weak HF solutions and the only conclusion, for the present, is that "antihemophilic globulin" lacks the accelerator effect.

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[15] Ware, A. G., Guest, M. M., and Seegers, W. H., *Am. J. Physiol.*, 1947, **150**, 68.

[16] Loomis, E. C., and Seegers, W. H., *Am. J. Physiol.*, 1947, **148**, 563.

We have shown elsewhere[12] that HF contains a protease precursor which, when activated by streptokinase, evinces proteolytic (*e.g.*, fibrinogenolytic, etc.) and "thromboplastic" (aiding thrombin formation) properties, similar to other plasma protease preparations. The negative clot-lysis tests included in Table II, A, show that, for reasons of dilution and lack of activation such protease plays no part in the present results.

AcG has been studied in several types of experiment with completely negative results as regards any indication that it may be related to the plasma protease[17] system, whether as active enzyme or precursor, or as activator or inhibitor of added precursor, streptokinase, or active protease. It is an important point, therefore, that the aid which the "accelerator globulin" affords to thrombin formation has no demonstrable relation to the plasma protease system (*cf.* [1]).

It is also of interest to note that AcG has no significant "antihemophilic" activity, which, of course, is to be expected from the normal prothrombin clotting times obtained in hemophilia, indicating that hemophilic blood has adequate amounts both of prothrombin and the accelerator factor.

*Conclusions.* 1. There is a new factor, or group of factors, (Quick's "labile factor," Owren's "factor V," Ware, Guest and Seegers' "accelerator globulin"), to be recognized in the first phase of the blood-clotting system. At present it may be considered an "accessory" activator distinct from calcium, thromboplastin, and thromboplastic enzyme. 2. This factor is found abundantly in fresh plasma and prothrombin (purified by the usual methods, *e. g.* [13]), but deteriorates on "aging," independently of the prothrombin. High dilution may be necessary to demonstrate this. The addition of fresh AcG will restore the original potency of the prothrombin, but aged AcG loses this property. 3. AcG is not "antihemophilic" and so-called "antihemophilic globulin" is not able to restore the activity of aged prothrombin. 4. "Owren's disease,"[18] a specific hemorrhagic disorder associated with a plasma deficiency of this new factor, is, in all probability, a true clinical entity, which suitable tests can differentiate from "idiopathic hypoprothrombinemia," hemophilia, and other bleeding diseases.

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[18] Owren, P. A., *Lancet*, 1947, **252**, 446.

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### 16262

## Prothrombin Conversion Factor of Dicumarol Plasma.*

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Dicumarol *in vivo* is generally believed to cause a deficiency of plasma prothrombin. The therapeutic value of serum[1,2] in the treatment of cattle with "hemorrhagic sweet clover disease," a dicumarol-induced diathesis is therefore puzzling.

An investigation was conducted on dogs to which dicumarol was administered orally or

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* Abridgment of a portion of the thesis submitted by Dr. Owen to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Ph.D. in Medicine.

[1] Schofield, F. W., *J. Am. Vet. M. A.*, 1924, **64**, 553.

[2] Roderick, L. M., *Am. J. Physiol.*, 1931, **96**, 413.

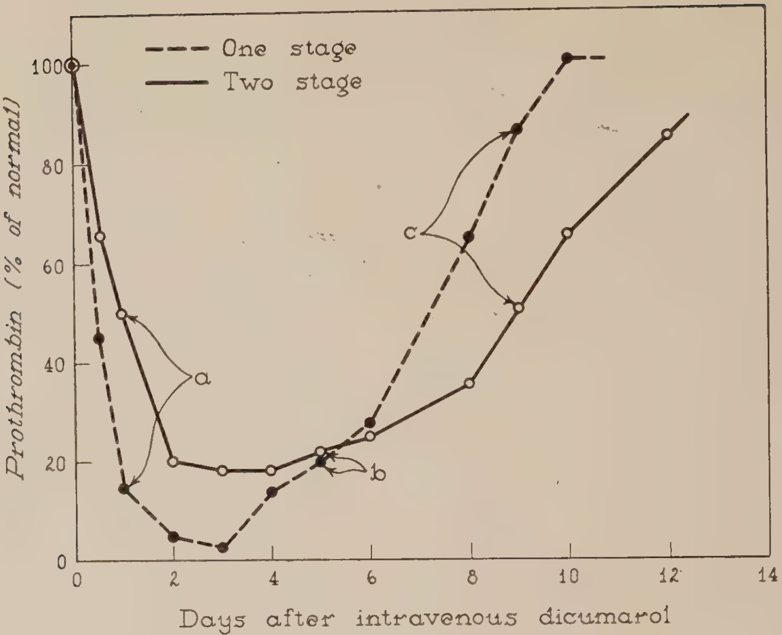

FIG. 1.

Changes of the prothrombin level as determined by the one-stage and the 2-stage methods after intravenous administration of dicumarol. For explanation of *a*, *b*, and *c* see text.

intravenously. Prothrombin determinations were performed by the one-stage test of Quick[3] and the 2-stage test of Warner, Brinkhous, and Smith.[4]

To increase the sensitivity of the Quick test, plasmas were studied at a number of dilutions, 0.3% fibrinogen being used as diluent; percentages were interpolated from the dilution curves of control samples.

In the 2-stage test, plasma is freed of fibrinogen by the addition of weak thrombin, which in turn is rapidly inactivated. The defibrinated plasma is diluted; calcium and thromboplastin are added and portions of the solution are mixed with fibrinogen, the clotting times being noted. Under standard conditions a coagulation time of 15 seconds denotes 1 unit of thrombin per milliliter, which is presumed to have arisen from 1 unit of prothrombin per milliliter. Since normal dog plasma, diluted about 300-fold, yields 1 unit of thrombin per milliliter, the undiluted plasma is said to contain about 300 units of prothrombin per milliliter. By determination of the concentration of thrombin at frequent intervals during its evolution not only the amount of thrombin formed but also the time for its formation are measurable.

As expected, the prothrombin level dropped after dicumarolization—the extent of the fall and its duration varying with the dose.[5] Fig. 1 shows the characteristic course in a female dog, weighing 8.5 kg, given 10 mg of dicumarol per kilo of body weight, by the intravenous route.

According to the Quick test the prothrombin fell more rapidly, to lower levels, but returned to normal sooner than by the 2-stage test. Thus, a sample of blood taken 24 hours after the drug had been administered (*a*) showed 50% prothrombin by the 2-stage test and 15% by the one-stage test. On the fifth day (*b*) the two methods were in close agreement. However, on the ninth day (*c*), with the level determined by the 2-stage test at 50% again, the prothrombin as determined by Quick's method had returned to almost normal levels.

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[3] Quick, A. J., Stanley-Brown, Margaret, and Bancroft, F. W., *Am. J. M. Sc.*, 1935, **190**, 501.

[4] Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

[5] Bollman, J. L., and Preston, F. W., *J. A. M. A.*, 1942, **120**, 1021.

Repetition of this experiment invariably yielded similar results. If smaller doses of dicumarol were employed, a point was reached (1 mg per kilo of body weight) at which the prothrombin, as measured by the 2-stage test, fell very little (to not less than 80%) while by the Quick test it dropped to a 40 or 50% level.

Study of the rate of conversion of prothrombin to thrombin seemed to indicate the cause of the discrepancy between the two methods. "Conversion times" represent the time required for conversion of 80% of the prothrombin into thrombin after addition of calcium and thromboplastin to plasma diluted to a prothrombin concentration of 1 unit per milliliter. It was found that when the Quick test yielded lower results than the 2-stage test, conversion of the prothrombin was delayed; when the tests agreed, conversion rates were normal; or, when the one-stage results were higher, conversion rates were accelerated above normal. Thus, in Fig. 1, at the predicumarol level (100%) conversion of 1 unit of prothrombin was 80% complete in 30 seconds; at (*a*) in 80 seconds; at (*b*) in 30 seconds; at (*c*) in 20 seconds.

In an attempt to discover the cause of the variable conversion rates of prothrombin it was noted that mixture of as little as one part of normal plasma with nine parts of slowly converting "dicumarol plasma" completely corrected the hypoconvertibility (from 80 seconds to 30 seconds) and likewise closed the gap between the one-stage and 2-stage tests. For example, at point (*a*) in Fig. 1, when 9 parts of the "dicumarol plasma" were mixed with one part of control plasma, the 2-stage test now read 52% (theoretically 55%); a Quick test on this mixture yielded 55% instead of the expected 24%. Further, addition of normal serum, free of prothrombin, thrombin, and fibrinogen, was equally corrective. Thus, some factor would seem to be present in normal serum, as well as in plasma, which can accelerate delayed convertibility.

Ammonium sulfate fractionation of normal dog serum revealed traces of the "conversion factor" in 25% and 33% saturation fractions, with the highest concentration at about 40 or 50% saturation. Dilution of serum and acidification to pH 5.3, followed by exposure to magnesium hydroxide, revealed little adsorption unless a great excess of the insoluble hydroxide was used. The factor, like prothrombin, is associated with the pseudoglobulins, but differs from prothrombin by being poorly adsorbed by magnesium hydroxide. Its deterioration at extremes of pH (5.5, 10.0), with moderate heating (50°C), and on exposure to air is much greater than that of prothrombin.

An analysis of dog plasma kept at 4°C for several weeks confirmed the findings reported in human and ox plasma;[6] that is, a rapid fall of prothrombin as measured by Quick's method, a slower fall as measured by the 2-stage test. Study of the rate of conversion of prothrombin of dog plasma kept at 4°C was made at intervals for a period of more than 60 days. Progressive hypoconvertibility was found to be the cause of the discrepancy between the methods. Ware, Guest and Seegers[6] have attributed the disparity to deterioration of fibrinogen. However, their data show incomplete correction when the fibrinogen of the plasma was replenished. While the fibrinogen must be adequate for performance of the Quick test, we find that correction of delayed conversion and of the difference between the prothrombin methods is possible only on addition of the conversion factor—such as in plasma, serum, or their pseudoglobulins.

There is a close resemblance between this conversion factor and Owren's factor V,[7] present in Seitz-filtered plasma, or the activators of Fantl and Nance[8] and of Ware, Guest, and Seegers,[9] as well as Quick's "prothrombin A."[10] The presence of the factor in serum might explain Schofield's[1] and Roderick's[2] therapeutic findings—findings difficult to understand if deterioration of prothrombin alone were present in cases of dicumarol or toxic sweet clover poisoning.

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[6] Ware, A. G., Guest, M. M., and Seegers, W. H., *Am. J. Physiol.*, 1947, **150**, 58.

[7] Owren, P. A., *Acta med. Scandinav.*, 1947 (suppl.), **194**, 327 pp.

[8] Fantl, P., and Nance, M., *Nature*, London, 1946, **158**, 708.

[9] Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

*Summary.* Evidence is presented that suggests the disappearance not only of prothrombin in dogs treated with dicumarol, but also of a factor important in the conversion of prothrombin to thrombin. Like prothrombin, this factor is associated with the plasma pseudoglobulins, but unlike prothrombin it is plentiful in serum. Addition of plasma, serum or serum pseudoglobulin corrects the delayed prothrombin convertibility of early dicumarolization or of stored plasma. The discrepancy between the one-stage and 2-stage methods of estimating prothrombin seems to reside in this conversion factor.

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[10] Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

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## 16263

# Immunological Studies of Newcastle Virus.

Reginald L. Reagan, Mary G. Lillie, Jean E. Hauser, and A. L. Brueckner.
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A California strain of Newcastle virus, No. 11,914, which was adapted to the Syrian hamster,[1] proved valuable as an immunizing agent in chickens against the virulent unmodified virus of this strain.[2] This successful immunization prompted continued experiments to study the relationship of the living modified virus as an immunizing agent, not only against the California strain but also against a Colorado strain, No. 8518, and a Connecticut strain, secured from the Bureau of Animal Industry, Washington, D.C.

Healthy 6-week-old New Hampshire Red chickens were used, and all birds were identified by wing bands. Serum from these chickens showed no neutralizing antibodies for Newcastle disease when tested by the chick embryo neutralization test prior to the start of the experiment. Three hundred fifty-two chickens were vaccinated by injecting 0.2 cc of a 10% suspension of virus-bearing hamster brain into the base of the wattle. Forty infected hamster brains of the eighty-second subculture were used for preparation of the vaccine, with physiological saline as the diluent. One hundred thirty-four unvaccinated chickens were placed with the vaccinated birds as room contact controls. All of the chickens were checked carefully each day. Table I shows the observations during the 28-day period prior to challenge.

Three groups were then set up, each consisting of vaccinated birds, unvaccinated room control and normal susceptible birds of corresponding age. Group I was challenged with the virulent chick embryo propagated California strain, No. 11,914, subculture 18. Group II was challenged with the virulent chick embryo propagated Colorado strain, No. 8518, subculture 8. Group III was challenged with the virulent chick embryo propagated Connecticut strain, subculture 8. The results obtained after challenge are shown in

TABLE I.
Evaluation of Chicken Deaths Following Vaccination, Prior to Challenge.

| | Vaccinated (352) | Room controls (134) |
|---|---|---|
| Coccidiosis | 10 | 1 |
| Newcastle | 1 | 0 |
| Smothered | 1 | 2 |
| Miscellaneous | 15 | 2 |

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[1] Reagan, Reginald, L., Lillie, Mary G., Poelma, Leo J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 136.

[2] Reagan, Reginald, L., Lillie, Mary, G., Poelma, Leo J., and Brueckner, A. L., to be published.

TABLE II.

Immunization of Chickens Against Newcastle Disease with Modified Newcastle Virus.
Route of vaccination: Base of wattle. Date of vaccination: 9/5/47
Route of challenge: Base of wattle. Date of challenge: 10/3/47
Challenge dose: 0.5 cc of given dilutions.
Challenge Virus (California Strain).

| Group I | Dilutions | | | | | | | | % | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | | $10^{-2}$ | | $10^{-3}$ | | $10^{-4}$ | | | |
| | S* | D* | S | D | S | D | S | D | D | S |
| Vaccinated | 18 | 2 | 15 | 5 | 17 | 3 | 17 | 3 | 16 | 84 |
| Unvaccinated contact controls | 2 | 8 | 7 | 3 | 4 | 6 | 3 | 7 | 60 | 40 |
| Normal controls | 3 | 7 | 5 | 5 | 6 | 4 | 3 | 7 | 57.5 | 42.5 |

* S—No noticeable symptoms.
D—Dead, also those showing Newcastle disease symptoms.

TABLE III.

Immunization of Chickens Against Newcastle Disease with Modified Newcastle Virus.
Route of vaccination: Base of wattle. Date of vaccination: 9/5/47
Route of challenge: Base of wattle. Date of challenge: 10/3/47
Challenge dose: 0.5 cc of given dilutions.
Challenge Virus (Colorado Strain).

| Group II | Dilutions | | | | | | | | % | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | | $10^{-2}$ | | $10^{-3}$ | | $10^{-4}$ | | | |
| | S* | D* | S | D | S | D | S | D | D | S |
| Vaccinated | 31 | 0 | 19 | 1 | 17 | 3 | 19 | 0 | 4.44 | 95.56 |
| Unvaccinated contact controls | 5 | 6 | 1 | 9 | 6 | 4 | 3 | 7 | 63.4 | 36.6 |
| Normal controls | 7 | 3 | 7 | 3 | 6 | 4 | 8 | 2 | 30.0 | 70.0 |

* S—No noticeable symptoms.
D—Dead, also those showing typical Newcastle disease symptoms.

TABLE IV.

Immunization of Chickens Against Newcastle Disease with Modified Newcastle Virus.
Route of vaccination: Base of wattle. Date of vaccination: 9/5/47
Route of challenge: Base of wattle. Date of challenge: 10/3/47
Challenge dose: 0.5 cc of given dilutions.
Challenge Virus (Connecticut Strain).

| Group III | Dilutions | | | | | | | | % | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | | $10^{-2}$ | | $10^{-3}$ | | $10^{-4}$ | | | |
| | S* | D* | S | D | S | D | S | D | D | S |
| Vaccinated | 13 | 1† | 20 | 1 | 19 | 0 | 19 | 1 | 1.4 | 98.6 |
| Unvaccinated contact controls | 9 | 1 | 8 | 2 | 18 | 0 | 10 | 0 | 6.25 | 93.75 |
| Normal controls | 9 | 1 | 6 | 4 | 8 | 2 | 8 | 2 | 22.5 | 77.5 |

* S—No noticeable symptoms.
D—Dead, also those showing Newcastle disease symptoms.
† Crushed.

Tables II, III, and IV, respectively. The challenge was injected into the base of the wattle in various dilutions on the twenty-eighth day after vaccination. The results of these tests are also given in Tables II, III, and IV, respectively.

Table II shows that the living hamster-adapted California strain provided good protection against the virulent egg-propagated California strain. The unvaccinated room controls and normal controls, challenged with the vaccinated chickens, showed approximately equal susceptibility.

In the case of the Colorado strain, No.

TABLE V.
Unchallenged Contact Control Chickens.

| Exposure strain | Vaccinated: Survivors c/o NC symptoms | Vaccinated: Newcastle symptoms | Unvaccinated: Survivors c/o NC symptoms | Unvaccinated: Newcastle symptoms |
|---|---|---|---|---|
| California | 30 | 0 | 15 | 5 |
| Colorado | 30 | 0 | 17 | 3 |
| Connecticut | 22 | 0 | 20 | 0 |

8518, the challenge virus was not as virulent as the California strain, as shown in Table III. The calculated amount of protection appears low, although only 4% of the vaccinated group failed to withstand the challenge. The unvaccinated room controls challenged with the Colorado strain showed greater susceptibility than normal controls challenged similarly.

The challenge virus of the Connecticut strain was of slightly lower virulence than that of the Colorado strain, as shown in Table IV. Since only one per cent of the vaccinated group showed typical Newcastle symptoms after challenge, the amount of protection is significant. The unvaccinated room control group showed much less susceptibility than the normal control birds in this group.

A contact exposure experiment was also conducted. Vaccinated and normal chickens of corresponding ages were placed with the control virus-challenged birds of the $10^{-1}$ and $10^{-2}$ dilutions of the California, Colorado, and Connecticut groups respectively, as shown in Table V. The results of this exposure are shown in the same table. None of the vaccinated birds succumbed. The contact exposure to Connecticut virus was not sufficiently great to cause disease in the normal unvaccinated birds. Comparatively few unvaccinated contact birds developed Newcastle disease in the California and Colorado groups. All unvaccinated chickens for this contact exposure were brought to the laboratory the day of challenge, at which time they were placed with their respective groups.

*Conclusion.* Hamster-adapted Newcastle virus of the 82nd subculture immunizes young susceptible chickens against virulent egg-adapted California strain No. 11,914 and against 2 less virulent strains, the Colorado strain No. 8518 and the Connecticut strain, subculture 8.

*Summary.* The pathogenicity of the 82nd subculture of the hamster-adapted California strain of Newcastle virus used as an immunizing agent was negligible in this experiment.

Through injection of the living hamster-adapted California strain No. 11,914, of Newcastle virus, young chickens were successfully immunized against the egg-adapted California, Colorado, and Connecticut strains. The modified Newcastle virus also produced exceptionally good protection in chickens to contact with chickens infected with all above-mentioned strains.

Susceptible birds, used as contact controls with birds injected with modified hamster-adapted Newcastle virus, failed to show clinical evidence of spread of this modified strain. There appeared to be some resistance to challenge injection in those birds challenged with the Connecticut strain, as compared with the normal unvaccinated control chickens of this group.

## 16264

# Phytopharmacological Experiments with Urethane.

DAVID I. MACHT.

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Ethyl carbamate or urethane has been known to pharmacologists for a long time. In fact, its physiological properties were first described by Schmiedeberg,[1] the father of modern pharmacology, who was the first to point out its narcotic properties.

At the beginning of the twentieth century it was quite extensively employed as an anesthetic for laboratory animals and as late as 1929 Macht and Hill[2] called attention to its usefulness for experimenting on small animals such as rats. Clinically, its usefulness was favorably reported towards the end of the nineteenth century as a hypnotic causing very little depression of the circulation and respiration.[3-7] The drug became popular in medical practice until the introduction of the powerful barbiturates which supplanted urethane on account of its rather weak and uncertain hypnotic effects on human beings. Nevertheless, the drug has been occasionally employed in psychiatric practice.

More recently, a new property of this organic chemical compound has been discovered by Haddow and Sexton, who investigated the action of urethane in connection with their studies on the influence of various drugs on the growth of malignant tumors. These studies revealed that while ethyl carbamate produced inconclusive effects on the growth of malignant cells, it did produce a very marked fall in the leucocyte count.

Prompted by this discovery, Paterson, Haddow, Thomas, and Watkinson[8] investigated the effects of urethane on the blood of leukemia and noted in 32 cases of human leukemia a decrease in the total white blood cell count and the return to a normal differential pattern of the white cells. Similar findings were reported by Engstrom, Kirschbaum, and Mixer[9] in their experiments with myelogenous leukemia in mice. More recently, similar studies were reported by Law concerning the effect of urethane on transplantable acute lymphoid leukemia, and further studies were described by Weir and Heinle.

Hirschboeck, Lindert, Chase, and Calvy[10] found little effect produced by urethane on malignant tumors but they did observe marked action of the chemical on leukemic tissues leading to satisfactory remissions in some human patients.

The explanation of this interesting property exhibited by ethyl carbamate is still not clear. Law, studying the effects of urethane on transplantable lymphoid leukemia, concludes that immature lymphoblasts are significantly decreased in leukemia patients and hemoglobin values remain at higher levels in patients treated with the drug. He also found that the growth of the local subcutaneous tumor masses was retarded.[11] Kirschbaum and Lu demonstrated that urethane decreased the number of mitotic figures in the marrow myeloid cells and reduced the total number of circulating white cells, especially myeloblasts, in transplanted myeloid leukemia of F strain mice, while Moeschlin[12] claims that in the treatment of leukemia with urethane there is noticed an elective inhibition of the karyokinesis of leukemic neoplastic cells.

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[1] Schmiedeberg, O., *Arch. f. exp. Path. u. Pharmokol.*, 1885, **20**, 203.

[2] Macht, D. I., and Hill, P. S., Jr., PROC. SOC. EXP. BIOL. AND MED., 1929, **26**, 871.

[3] Andrews, J. B., *Am. J. Insan.*, 1886, **43**, 256.

[4] Huchard, H., *Bull. gen. de therap.*, 1886, **110**, 103.

[5] Hübner, C., and Sticker, G., *Deutsche med. Wchnschr.*, 1886, **12**, 236.

[6] Sieveking, E. H., and Manifold, C. C., *Brit. M. J.*, 1886, **2**, 108.

[7] Binet, P., *Rev. med. de la Suisse Rom.*, 1893, **13**, 540, 628.

[8] Paterson, E., Haddow, A., Thomas, I., and Watkinson, J. M., *Lancet*, 1946, **1**, 677.

[9] Engstrom, R. M., Kirschbaum, A., and Mixer, H. W., *Science*, 1947, **105**, 255.

[10] Hirschboeck, John S., Lindert, M. C. F., Chase, Jules, and Calvy, Thomas L., *J. A. M. A.*, 1948, **136**, 90.

[11] Law, L. W., *Proc. Nat. Acad. Science*, 1947, **33**, 204.

The present author for the past 25 years studied the action of various chemicals, drugs, and toxins on plant cells as compared with animal cells. These studies which have been carried on in a quantitative experimental fashion on various plants and especially on the root growth of *Lupinus albus* seedlings have been described in detail.[13,14] It was deemed worthwhile, therefore, to examine the effects of urethane by phytopharmacological methods and the results are reported briefly. This was deemed especially worthwhile because of the recent announcement by Giles that radioactivity produces marked changes in plant cells.[15]

*Method.* The biological criterion employed was the elongation or growth of the well-defined straight roots of Lupinus seedlings, studied by the author's phytopharmacological methods, details of which have been published. The procedure was briefly as follows:

Seeds of *Lupinus albus,* large variety, were soaked in tap water overnight and planted in finely ground sphagnum moss containing adequate moisture. After germination, the growth of selected seedlings with roots 35 to 50 mm long was studied in a solution consisting of equal parts of distilled water and Shive physiological saline.[16] At least 20 seedlings, placed in hard-glass test tubes, were employed for the control solution and for each drug solution, in the different concentrations used. The sharply defined straight roots were measured at the beginning and end of every experiment, and the ratio of growth of the seedling roots in the various drug solutions is expressed as a percentage of their growth in the control solution according to the formula,

$$\text{Index of growth} = \frac{X}{N} \times 100$$

in which N represents average growth of controls and X the average growth in the drug solution. All of the seedlings were allowed to grow for 24 hours in the dark at 15°C.

*Results.* In Table I are shown the results obtained with different concentrations of urethane on the growth of *Lupinus albus* seedlings studied in a manner described above. It will be seen that normal seedlings are very little affected by the drug. Even solutions 1:1000 are inhibited only slightly, the index of growth being on the average above 90%.

A very different effect of urethane was obtained by the use of seedlings raised in a different manner. The present writer has been making studies on the comparative reaction of normal seedlings on the one hand and so-called vernalized or "yarovized" seedlings of *Lupinus albus.* Yarovization is a term introduced by Russian plant-physiologists to describe a phenomenon produced by exposing plants to extreme variations of temperature.[17] A simple and efficient method for obtaining yarovized seedlings is to expose them to quite low temperature but not such as would freeze the plant. Such plants obviously are markedly changed in their metabolism and the author has found that such seedlings may respond differently to certain blood sera and drugs from the responses given by normal seedlings, even though both the normal and the previously yarovized plants are allowed to grow at normal temperatures when the phytopharmacological experiment is performed. In the present research, Lupinus seedlings were germinated in sphagnum moss and when they were 2 days old, the jar with the young seedlings was placed in a refrigerator for 24 hours at 42°F (5.5°C). These seedlings were then employed simultaneously with other seedlings reared at a normal temperature of 70° and exposed to the action of various concentrations of urethane. Both sets of plants were placed in a low tempera-

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[12] Moeschlin, Sven, *Experientia,* 1947, **3**, 195.

[13] Macht, D. I., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 217.

[14] Macht, D. I., and Macht, M. B., *J. Lab. and Clin. Med.,* 1941, **26**, 597.

[15] Giles, Norman F., Jr., *Proc. Nat. Acad. Sci.,* 1947, **33**, 283.

[16] Shive, J. W., *Physiol. Rev.,* 1915, **1**, 327.

[17] Miller, E. C., *Plant Physiology,* 2nd Edition, 1938.

TABLE I.
Influence of Urethane on Growth of Seedlings.

| Normal Plants. | | Yarovized Plants. | | Irradiated Plants, 500 r | |
|---|---|---|---|---|---|
| Urethane | Phytotoxic index, % | Urethane | Phytotoxic index, % | Urethane | Phytotoxic index, % |
| 1:10,000 | 105 | 1:100,000 | 86 | 1:100,000 | 92 |
| 1: 5,000 | 100 | 1: 75,000 | 76 | 1: 50,000 | 87 |
| 1: 2,000 | 100 | 1: 60,000 | 70 | 1: 25,000 | 65 |
| 1: 1,000 | 98 | 1: 50,000 | 58 | 1: 10,000 | 60 |
| 1: 500 | 90 | 1: 10,000 | 49 | 1: 5,000 | 50 |
| 1: 250 | 72 | 1: 5,000 | 36 | 1: 1,000 | 25 |
| 1: 100 | 60 | 1: 1,000 | 24 | | |
| | | 1: 500 | 17 | | |

ture thermostat for 24 hours at 60°F (15°C) and the influence of the drug on root growth was compared in the usual manner.

It was found that vernalized plants were extremely sensitive to the effects of urethane, whereas normal seedlings were very little affected by concentrations of 1:1000. The vernalized seedlings were sensitive to solutions of as little as 1:100,000. This is well illustrated in Table I.

A third method of studying urethane on what may be termed abnormal plant cells was by the employment of seedlings treated with deeply penetrating X-rays. Seeds of *Lupinus albus* were germinated in sphagnum and when 2 days old were radiated with X-rays filtered through a composite filter consisting of 1 mm of aluminum and 2 mm of copper. The apparatus used was a therapy machine operated on 200 Kv on a current of 20 ma, the plants being at a target distance of 50 cm. The dosage employed for the most part in the present experiments was 500 r. The results obtained are shown in the third column of Table I. It will be noted that such irradiated seedlings were also rendered much more sensitive to the effects of ethyl carbamate than normal seedlings.

*Comment.* There is considerable literature dealing with the inhibitory or suppressive action of urethane, phenyl carbamate, and other carbamates on the growth processes of bacteria, protozoa, and various animal tissues, as well as plant tissues. Templeman and Sexton[18] studied the effects of such compounds upon cereals and other plant species. Lefèvre[19] studied the effects of urethane on wheat seedlings, oats, barley, and rye. Both of the above investigators noted cytological alterations, blocking of mitosis, and other profound changes in the plant soil protoplasm produced by the drugs, not unlike those produced by colchicine. As far as the author is aware, however, no pharmacological studies were reported in which abnormal seedlings were compared with normal ones in respect to their reactions to urethane. The present findings, therefore, are of considerable interest, and are of particular interest in connection with the effects produced by urethane on malignant tumor cells and on leukemia blood cells of animals as compared with normal animal tissue cells. It is not at all uncommon in pharmacology to find a difference in reaction to drugs or poisons between normal animal cells and pathological animal tissues.[20] The same has been shown by the present author to be true of plant cells and is strikingly illustrated in the present investigation which reveals such a marked difference in response to urethane by the normal Lupinus seedlings and seedlings which have been yarovized on the one hand and irradiated on the other. These studies should encourage further investigations on the effects of various chemicals and drugs on abnormal cells both of animals and plants with a view of finding possibly some valuable agents that may inhibit the growth of malignant issues.

*Summary.* Solutions of ethyl carbamate or urethane were studied in different concentrations as regards their influence on root growth of *Lupinus albus* seedlings. It was found that whereas normal seedlings are very little affected by the drug, other seedlings of an abnormal type were very sensitive to urethane. Specifically, it was found that yarovized or vernalized seedlings are extremely sensitive to even dilute solutions of the drug and the same was found to be true of seedlings which were irradiated with hard X-rays in doses of 500 r.*

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[18] Templeman, W. G., and Sexton, W. A., *Nature*, 1945, **156**, 630.

[19] Lefèvre, J., *C. R. Acad. Sci. Paris*, 1939, **208**, 301.

[20] Macht, D. I., and Ting, G. C., *J. Pharm. and Exp. Therap.*, 1921, **18**, 111.

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* By courtesy of Dr. Marcus Ostro, Radiologist in Chief, Sinai Hospital, Baltimore.

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### 16265

## An Easily Assembled Pulse-Frequency Recorder.

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Continuous, automatic, kymographic recording of pulse frequency conserves time, recording paper, and workers' patience. The device to be described is easily assembled with ordinary laboratory tools and supplies.

As shown in Fig. 1, a heart-lever fulcrum ($F_1$) is equipped with a tight-fitting, hollow laboratory straw (carrying lever, $L_1$) bearing an adjustable counterpoise (WE) on the short arm, and the fulcrum ($F_2$) of a second straw (contacting lever, $L_2$) on the long arm. The counterpoise is a weighted heart-lever fulcrum. The fulcrum of the contacting lever is a piece of aluminum (lever) wire, wound about and cemented (Duco) to the carrying lever, and passing as a bearing pin in a horizontal hole through the contacting lever. Into one end of the contacting lever is cemented a soldered-wire slip-yolk (Y) which engages the cross (recording) wire (M) of an ordinary recording mercury manometer. Into the other end is cemented a platinum wire which makes contact (C) with a drop of mercury in a tiny platinum-foil cup soldered onto the fulcrum block of the carrying lever. The long arm of the carrying lever is extended with an aluminum wire shaped (*a*) to serve as an adjustable stop (ST) for the contacting lever, and (*b*) as a lateral friction surface of the carrying lever against a lightly contacting, silk thread (FR) rendered slightly taut by the tension of a rubber band at one end. Contact C closes a circuit between the grounded and insulated (INS) binding posts ($BP_1$ and $BP_2$) on the insulation-sheathed supporting rod (SU). The conducting wire (WI) from the platinum wire must be fine and flexible.

The contacting lever is balanced on its fulcrum by a suitable combination of fulcrum position and lengths of wires inserted into its ends. The entire system is then balanced on the carrying fulcrum by adjustment of counterpoise, WE. Engagement with the manometer thus has no significant influence on the latter's mean recording position.

To facilitate adjustments, universally adjustable clamps are used for mounting the device proper and the rods between which the friction thread is strung. The stop (ST) for the contacting lever is adjusted by bending so that the lever's freedom of excursion does not exceed the smallest systolic excursion of the manometer apt to be experienced (short of terminal cardio-vascular failure). The light tension of the friction thread is adjusted to just hold the carrying lever at the position to which it is brought by the excess of the manometer excursion beyond the contacting lever's short range. Automatic compensation thus occurs to changing mean and pulse pressures.

In present use, this contacter activates, through a standard-type electronic relay, a modification of the "counter-recorder" previously described.[1] The modification consists of branching the power end of the recording

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[1] Winder, C. V., and Moore, V. A., *J. Lab. and Clin. Med.*, 1945, **30**, 894.

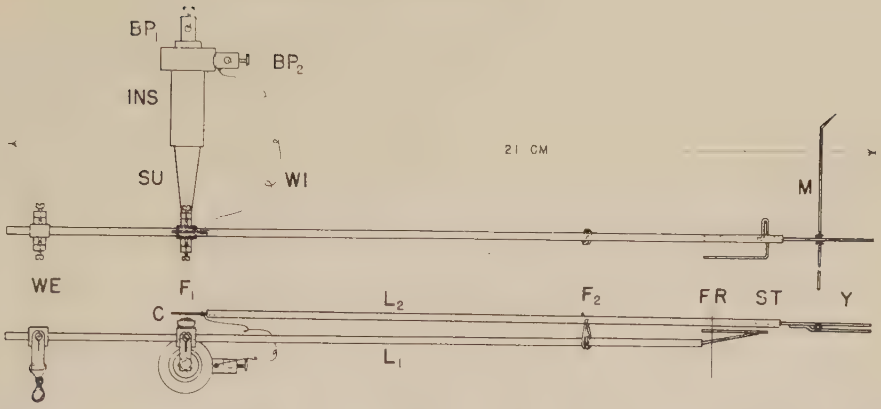

Fig. 1.
Contacting lever system. See text.

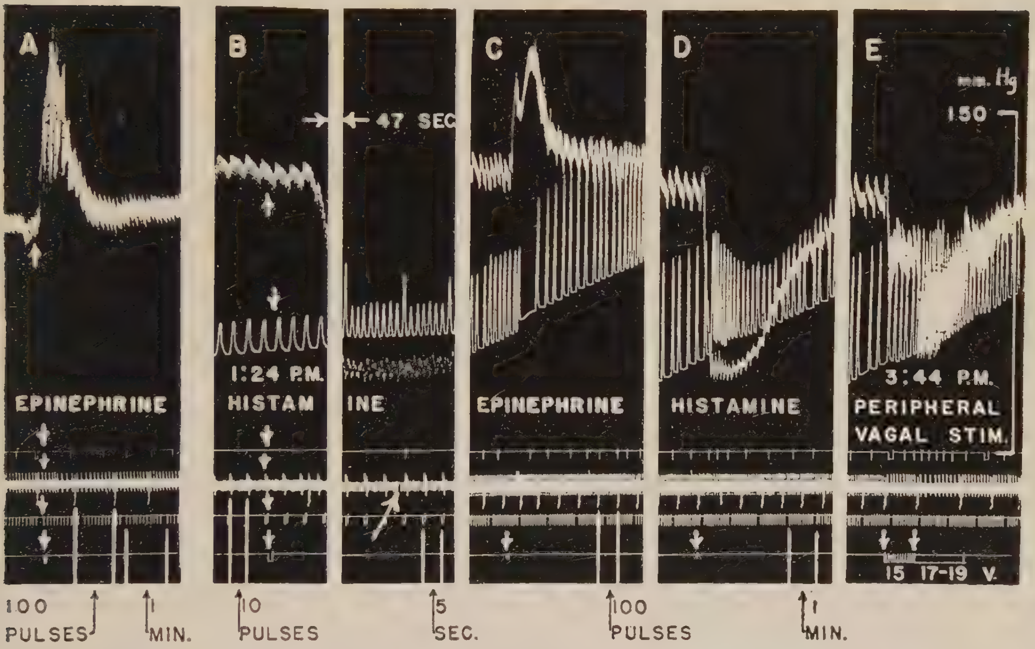

Fig. 2.
Illustrations of pulse-frequency recording under various conditions and recording speeds. *A* is from one phenobarbitalized dog, *B* to *E* from another. Small vertical arrows indicate simultaneous points. Vagus nerves were cut between *B* and *C*. Recordings from below upward are signal, time, pulse frequency, respiratory minute volume, spirometer, and femorál arterial manometer engaging the contacting lever. Large diagonal arrow indicates failure of one individual pulse to register (see text).

lever into three tines which are displaced, respectively, by (a) the rachet, (b) a notch in the units-wheel, and (c) the lug on the tens-wheel of the counter mechanism. On a single record, individual pulses, tens, and hundreds are thus characterized by suitable amplitudes and/or directions of lever-movement. This summating recorder does not require calibration, and is more compact and more easily devised than a Zeitschreiber.[2–6] The latter, equally well adaptable to

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[2] Fleisch, A., in Abderhalden, E., *Handbuch der biologischen Arbeitsmethoden*, Urban & Schwarzenberg, Berlin and Wien, 1935, Abt. V, Teil 8, 1935, p. 905.

[3] Gesell, R., *Science*, 1934, **79**, 275.

[4] Gaddum, J. H., and Kwiatkowski, H., *J. Physiol.*, 1938, **94**, 87.

the present contacter, does not require exact reference to a simultaneous record of time, indicates instantaneous changes in frequency, and provides a ready-made graph of the (reciprocal) frequency course. Junkmann's[7] original Impulszähler falls into the latter category except for *direct* frequency plotting and for lack of instantaneous information.

As illustrated in Fig. 2, with optimal adjustment, the systolic excursions of the manometer are selectively registered during widely and abruptly varying patterns of respiratory pressure waves, pulse pressure, and mean pressure. The limits of fidelity are those of the mercury manometer, as illustrated by the individual unregistered systole in Fig. 2B (arrow), where a very low pulse oscillation was masked by relatively vigorous respiratory waves during histaminic hypotensive dyspnea.

The principle of automatic compensation to varying hemodynamics, fully present in the contacting instrument of Genuit and Ruppert[6] which inspired the present simplification, was lacking in Fleisch's Pulspelotte and partially present in such manner as to operate against sensitivity in Nyboer's[8] contacter. Electronic cardiotachometers employing the cardio-electric R-wave are, of course, inherently independent of hemodynamics.

It will be apparent that the contacter described is adaptable to many oscillatory phenomena, with provision of a simple engagement for the contacting lever.

*Summary.* A simple contacting lever, mounted on a carrying lever and engaged with a mercury-manometer cross-wire, selectively registers systolic pulsations. It is used to activate, through a relay, a simple counter-recorder,[1] improved. Individual pulses, tens, and hundreds (etc.) are characterized by amplitude and/or direction of excursion of a single recording lever. Operation and limitations are illustrated.

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[5] Druckrey, H., and Uhlig, P., *Arch. f. exp. Path. u. Pharmakol.*, 1941, **198**, 74.

[6] Genuit, H., and Ruppert, H., *Arch. f. exp. Path. u. Pharmakol.*, 1943, **200**, 684.

[7] Junkmann, K., *Arch. f. exp. Path. u. Pharmakol.*, 1927, **120**, 314.

[8] Nyboer, J., *Am. J. Physiol.*, 1933, **106**, 204.

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### 16266

# Alkaline Phosphatase Activity and Basophilia in Hepatic Cells Following Administration of Butter Yellow to Rats.*

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A cytochemical study[1] has been reported previously of the activity of phosphatase (at pH 5.0) in the cytoplasm of motor neurons during their regeneration. A well-defined increase was found to be associated with the dissolution of cellular basophilic material, chromatolysis, and its regeneration, chromatogenesis.

The alterations in phosphatase activity previously described were in cells which do not divide. It was desirable, therefore, to extend the observations to include regenerating cells in which mitosis can be investigated.

There are presented herewith the results of a study of alkaline phosphatase activity as correlated with basophilia in the hepatic cells of rats fed butter yellow (*p*-dimethylaminoazobenzene) in a diet which renders the liver of the animal susceptible to consequent cancer production.

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* Presented at the Fourth International Cancer Research Congress, St. Louis, September 6, 1947. A rather similar study was reported by N. Bucher and A. Glinos at this Congress.

† This work was supported by a Fellowship Grant of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

[1] Bodian, D., and Mellors, R. C., *J. Exp. Med.*, 1945, **81**, 469.

A large part of the cytoplasmic basophilic substance is contained in prominent structural entities, called Nissl bodies in the case of the nerve cell. These structures have been shown to consist of a protein matrix with a basophilic component which can be removed by ribonuclease activity.[2,3] They absorb ultraviolet light at 2600 Å,[3,4] and do not react by Feulgen's method. It can be concluded, therefore, that they contain nucleic acid of the ribose type.

Opie[5] described the mobilization of basophilic material in the cytoplasm of liver cells in which degenerative, regenerative, and proliferative changes had been induced by butter yellow. This material was shown by Opie and Lavin[6] to have the characteristics of ribonucleic acid.

The demonstration of alkaline phosphatase in large granules separated from the cytoplasm by Claude[7] and in residual chromosomes isolated from the nucleus by Mirsky and Ris[8] indicates an association of this enzyme, or group of enzymes, with structural and functional components of the cell.

*Methods.* Thirty-three albino rats (Sherman strain) were offered a diet of unpolished rice supplemented with fresh carrots. Butter yellow, *p*-dimethylaminoazobenzene,[9] was included at a level of 0.06%. Six normal rats of comparable age and weight, offered the same diet but without butter yellow, were studied as controls. At approximately weekly intervals from 7 to 258 days, the animals were killed and studied histologically. The weights at death were from 85 to 380 g.

Thin slices of liver were fixed immediately in ice-cold acetone, imbedded in paraffin, and cut in serial sections. The method of Gomori[10] was employed for the histological demonstration of alkaline phosphatase activity at pH 9.0, temperature 37°C, and period of incubation from 1 to 3 hours. A variety of substrates were used: glycerophosphate, fructose diphosphate, adenylic acid, and ribonucleic acid (from yeast). Adjacent sections were stained by a variety of technics, the most useful of which was that of Giemsa (Wohlbach's modification).[11] In addition, sections were studied for basophilia, when stained with methylene blue and toluidine blue under controlled conditions,[12] before and after incubation in a solution of purified ribonuclease,[‡,13] (enzyme concentration 0.1 mg/cc, pH 6.75, temperature 58°C, duration 2 hours) and desoxyribonuclease,[14] (enzyme concentration 0.1 mg/cc, pH 6.75, Mg ion 0.003 M., temperature 37°C, duration 2 hours).

*Results.* Although acetone fixation is not ideal, basophilic bodies were prominent in the cytoplasm of the normal rat liver cells stained by Giemsa's procedure. (Fig. 1A). Alkaline phosphatase activity was uniformly slight in both the cytoplasm and the nucleus of these cells (Fig. 1B).

After the administration of butter yellow, however, degeneration with chromatolysis and regeneration with chromatogenesis were present in the cytoplasm essentially as described by Opie.[5]

Early focal chromatolytic changes were demonstrable after 24 days, and were prominent after 45 days, when some cells had lost much or all of their cytoplasmic basophilia and, in some instances, the staining properties of their nuclei as well. After 51 days, basophilia as evidence of chromatogenesis was seen in some cells, although advanced chromatolysis was still predominant. At 95

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[2] Brachet, J., *C. R. Soc. biol.*, 1940, **133**, 88.

[3] Gersh, I., and Bodian, D., *J. Cell. and Comp. Physiol.*, 1943, **21**, 253.

[4] Landstrom, H., Caspersson, T., and Wohlfart, G., *Z. mikr-anat. Forsch.*, 1941, **49**, 534.

[5] Opie, E. L., *J. Exp. Med.*, 1946, **84**, 91.

[6] Opie, E. L., and Lavin, G. I., *J. Exp. Med.*, 1946, **84**, 107.

[7] Claude, A., *A. A. A. S. Research Conference on Cancer* (1944), Washington, D.C., A. A. A. S., 1945, 223.

[8] Mirksy, A. E., and Ris, H., *J. Gen. Physiol.*, 1947, **31**, 7.

[9] Sugiura, K., and Rhoads, C. P., *Cancer Research*, 1941, **1**, 3.

[10] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

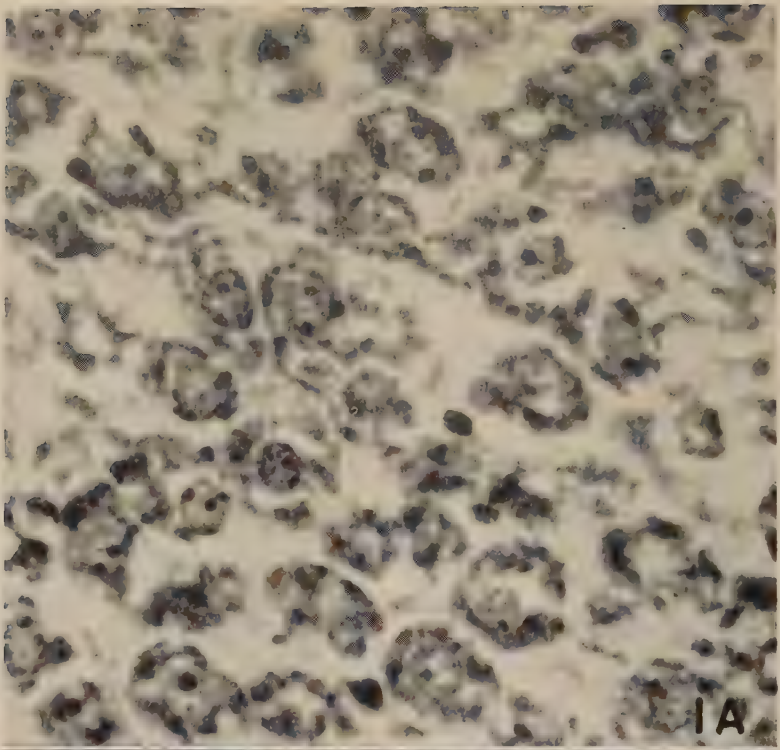
[11] Mallory, F. B., *Pathological Technique*, Philadelphia, W. B. Saunders Co., 1938, 195.

[12] Dempsey, E. W., and Singer, M., *Endocrinol.*, 1946, **38**, 270.

[‡] Kindly supplied by Dr. M. Kunitz.

[13] Kunitz, M., *J. Gen. Physiol.*, 1940, **29**, 15.

[14] McCarthy, M., *J. Gen. Physiol.*, 1946, **29**, 123.



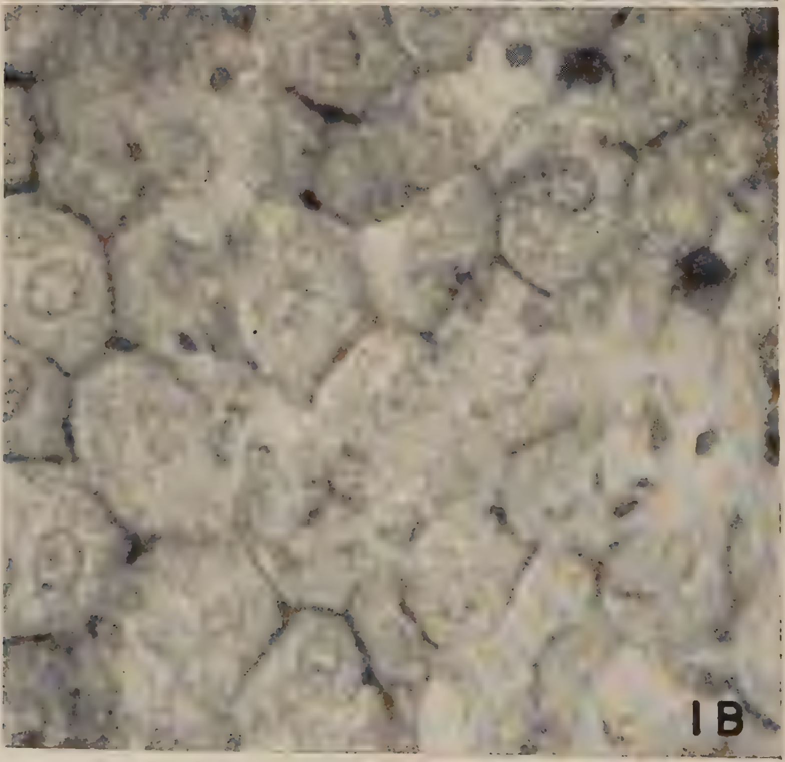


FIG 1A. Normal hepatic cells containing cytoplasmic basophilic material. Acetone fixation. Paraffin section 4 microns. Giemsa (Wolbach's). 500 ×.

FIG. 1B. Normal hepatic cells with slight alkaline phosphatase activity. More conspicuous activity in biliary capillaries. Acetone fixation. Paraffin section 5 microns. Gomori's method. Glycerophosphate substrate, pH 9.0, 3 hours' incubation at 37°C. 500 ×.

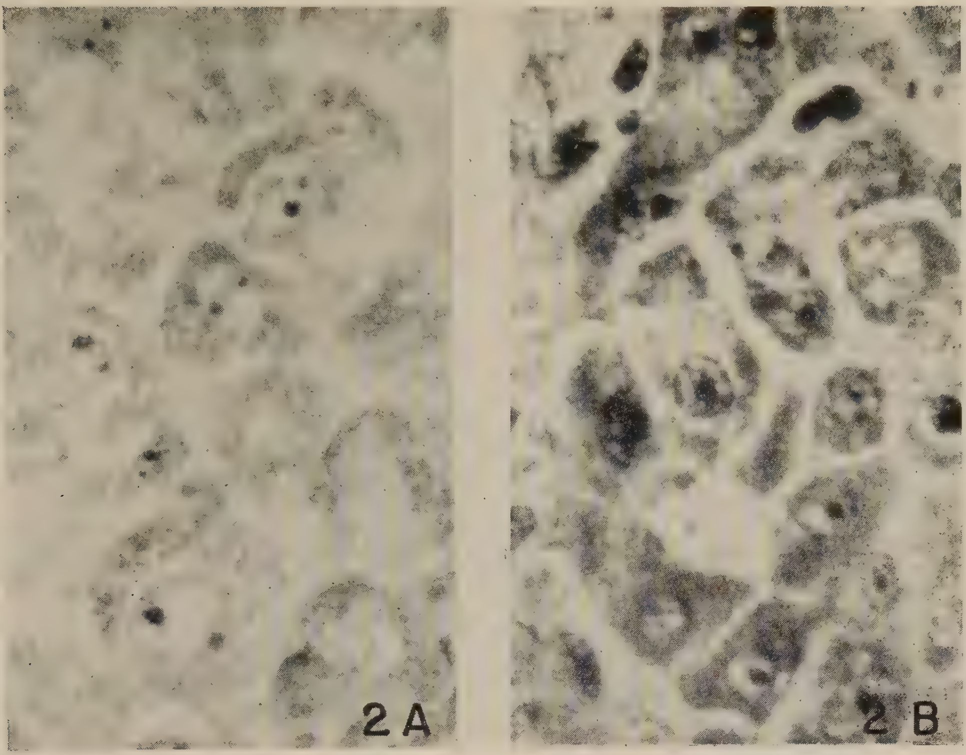


FIG. 2A. Degeneration and chromatolysis in hepatic cells after 95 days of butter yellow administration. Acetone fixation. Paraffin section 4 microns. Giemsa (Wolbach's). 500 ×.
FIG. 2B. Same section as 2A. Focus of regeneration and chromatogenesis in hepatic cells, with prominent cytoplasmic basophilia.

days, cytoplasmic basophilia was intense in foci of regeneration and hyperplasia (Fig. 2B) in sharp contrast to the faint basophilia of cells with persistent evidence of degeneration and necrosis (Fig. 2A). After 197 days, focal basophilic regeneration and hyperplasia were associated with hepatoma, cholangioma, and adenocarcinoma.

A slight tendency toward decreased alkaline phosphatase activity at pH 9.0 was present in association with the hepatic chromatolysis (Fig. 3A). In contrast, an increased activity was frequently present, both in the nucleus (nuclear membrane, chromatin, nucleolus, dividing chromosomes) and to a lesser degree in the cytoplasm in foci of basophilic hyperplastic and proliferating cells (Fig. 3B). This conclusion was established by direct comparisons of sections stained for alkaline phosphatase activity with adjacent sections stained by the Giemsa procedure. In these comparisons, it was clear that, although a good correlation could be drawn between basophilic hyperplasia of parenchymatous cells and a distinct increase in phosphatase activity, the findings were not absolutely uniform.

Observations of cells other than parenchymatous under the conditions of these experiments were of interest. In the epithelium of the bile ducts, no significant alteration in phosphatase activity occurred either during proliferation or actual hyperplasia. Intense, although not necessarily increased, phosphatase activity distinguished mast cells, sometimes present in abundance in the connective tissue around proliferating bile ducts. Conspicuous activity was occasionally present in the sinusoidal endothelium and usually present in the endothelium of the small blood vessels. Moderate activity was in evidence in proliferating fibroblasts in the stroma of cirrhosis and cholangiofibrosis.

In hepatoma, cholangioma, and metastasizing adenocarcinoma, the phosphatase activ-

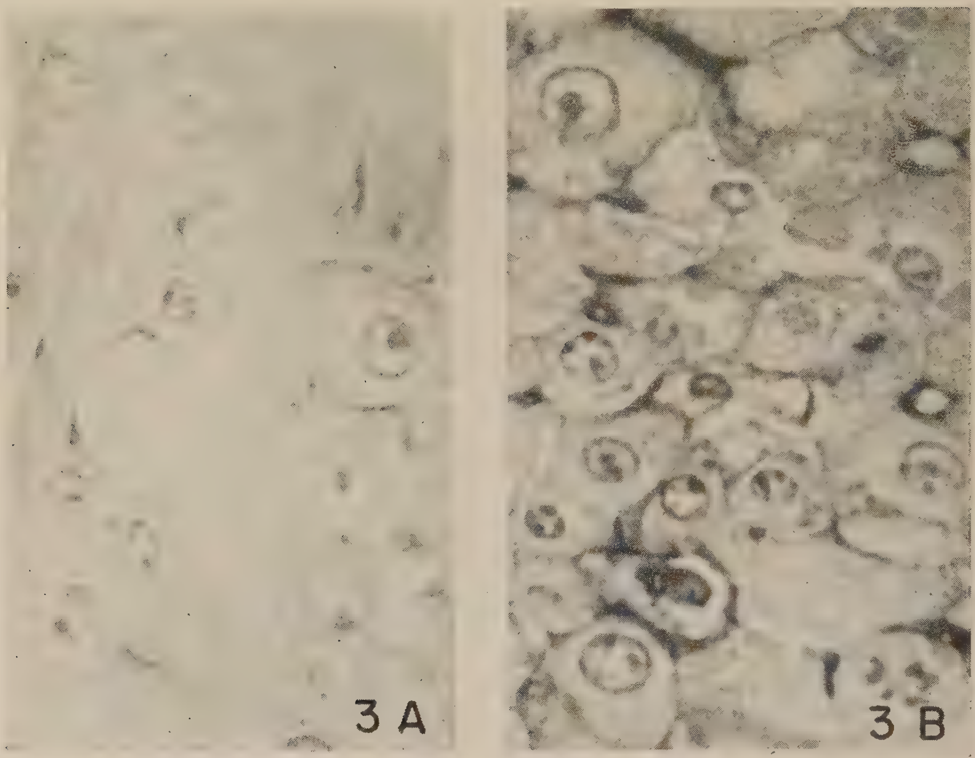


Fig. 3A. Section adjacent to 2A. Same area of degenerating and chromatolytic hepatic cells as shown in 2A, with very little alkaline phosphatase activity. Acetone fixation. Paraffin section 4 microns. Gomori's method. Glycerophosphate substrate, pH 9.0, 3 hours' incubation at 37°C. 500 ×.

Fig. 3B. Same section as 3A. Same focus of regeneration and chromatogenesis as shown in 2B, with increased alkaline phosphatase activity in nucleus (membrane and chromatin), chromosomes, and, to a lesser extent, in cytoplasm of hepatic cells. Activity is conspicuous also in a bile capillary.

ity, variable and focal in distribution, occurred in the tumor cells, particularly in those which appeared to be degenerated or to be associated with necrotic debris.[15]

*Summary aand Conclusions.* As with the reformation of Nissl substance in the motor neuron, the resynthesis of nucleic acid in the cytoplasm of the regenerating hepatic cell, injured by butter yellow, is accompanied, during certain phases of the reaction at least, by an enhanced activity of alkaline phosphatase. The cellular distribution of the alteration is principally cytoplasmic in the nerve cell, with the optimum pH in the acid range, and nuclear in the hepatic cell, with the optimum pH in the alkaline range.

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[15] Kabat, E. A., and Furth, J., *Am. J. Path.*, 1941, **17**, 303.

### 16267

## Diagnosis of Pregnancy by Cytologic Criteria in Catheterized Urine.*

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Pregnancy causes marked proliferation and growth in the epithelium of the vagina and the cervix.[1] This results in characteristic cytologic changes, which permit the diagnosis of pregnancy through the microscopic examination of fluid aspirated from the vagina. The use of the vaginal smear in diagnosing pregnancy was advocated by me in 1925.[2]

The extreme growth of the epithelium of the vagina and the cervix during gestation is accompanied by a considerable increase in the secretion of glycogen. Glycogenic cells become thus very prominent in the vaginal smear of pregnancy. The most typical of these cells are the so-called "navicular" cells,[3] which have a definite diagnostic value. Their differentiation from corresponding cells of the normal cycle is possible through certain criteria such as enlargement and elongation of their nuclei and thickening of their surface membranes.

The vaginal smear method of diagnosing pregnancy, though morphologically sound, did not prove to be of great practical value as a routine diagnostic procedure. The identification of the "navicular cells of pregnancy" requires great experience and is not always dependable. It is often difficult to differentiate these cells from other forms of navicular cells found during the normal menstrual cycle or in certain types of secondary amenorrhea. Furthermore, other factors, such as intercourse or bacterial and parasitic infections may distort the vaginal smear picture, thus impairing its diagnostic value.

In the course of studies of smears prepared from urine sediment for the purpose of detecting cancer cells,[4,5,6] I have been impressed by the greater cytologic uniformity of such smears in comparison with vaginal smears. The normal exfoliation in the urinary tract is much scantier than in the female genital tract and the variety in the types of desquamated cells is much more limited. The appearance of any significant cytologic changes can thus be noted much more readily in the "urine sediment" than in the vaginal smear.

The effect of the estrogenic hormone upon the cellular make-up of the urine sediment smear may be cited as an example. This effect is noticeable in the urine of women as well as of men. I first observed it in the case of a man receiving estrogenic therapy for a carcinoma of the prostate.[5] It consists in the appearance of squamous acidophilic cells with a pyknotic nucleus and cytoplasmic granules greatly resembling the cells found in the vaginal smear either normally during the follicular phase or after estrogenic therapy. The recognition and identification of these cells is indeed easier and less equivocal in the "urine" than in the "vaginal" smear. This is because the vaginal smear contains almost invariably other non-specific squamous acidophilic small-nucleated cells which complicate the cytologic picture. The urine sediment smear may thus prove to be more accurate in assaying estrogens than the vaginal smear. The urine of women should be obtained by catheterization in order to prevent the admixture of vaginal cells.

More recently, my attention was directed

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* Aided by a grant from the Commonwealth Fund.

[1] Stieve, H., *Z. f. mikrosk.-anat. Forsch.*, 1925, **3**, 3.

[2] Papanicolaou, G. N., PROC. SOC. EXP. BIOL. AND MED., 1925, **22**, 436.

[3] Papanicolaou, G. N., *Am. J. Anat.*, 1933, **52**, 3 (Supplement), 519.

[4] Papanicolaou, G. N., and Marshall, V. F., *Science*, 1945, **101**, 2629, 519.

[5] Papanicolaou, G. N., *J. Am. Med. Assn.*, 1946, **131**, 372.

[6] Papanicolaou, G. N., *J. Urol.*, 1947, **57**, 375.

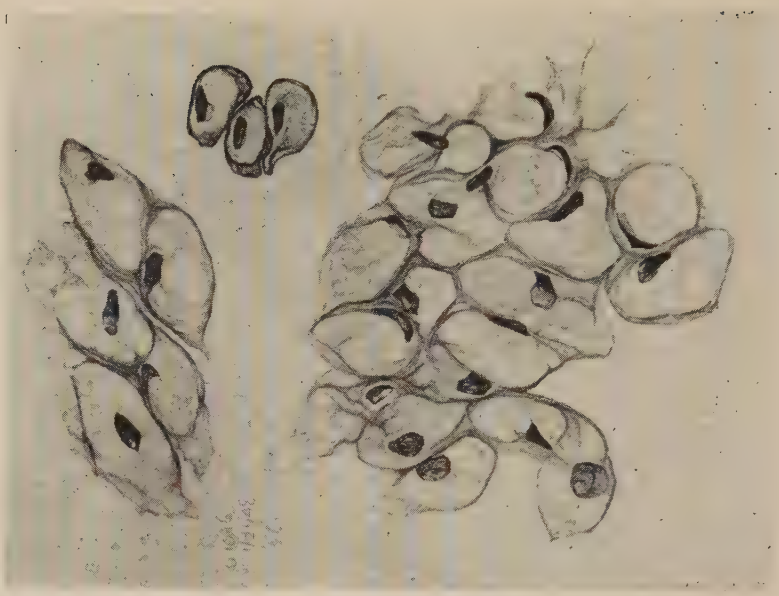

FIG. 1.
Photograph of drawings of cells of the navicular type found in smears prepared from the urine of pregnant women. × 400.

toward the cytologic changes occurring in the urine of pregnant women. For this study catheterized urine was used, since voided urine is always contaminated with cervical, vaginal, and vulval cells. The pure catheterized urine contains normally a relatively small number of exfoliated cells, most of which are of the transitional type. The variety of cell types is limited. Leucocytes are, as a rule, scanty, except in cases of bacterial or parasitic infections of the urinary tract, which are much less frequent than those of the vagina and the cervix. Histiocytes are also relatively rare.

In the pregnancy urine, there is an apparent increase in the total number of desquamated cells. The cells appear singly or in clusters. Some of these acquire a characteristic form closely resembling that of the navicular cells found in the vaginal smear of pregnancy (Fig. 1). Here again, under the effect of the hormonal factors of pregnancy, the cytology of the urine sediment smear acquires a great similarity with that of the vaginal smear. Many of the cells show pronounced vacuolation and a displacement of the nucleus toward the periphery or one of the poles of the cell. The presence of glycogen within the vacuoles may be demonstrated by special staining. There is, thus, evidence of an increased production of glycogen during gestation in the epithelium of the bladder, parallel to that of the epithelium of the cervix and the vagina. Leucocytes are, as a rule, inconspicuous. In general, the urine sediment smear shows a simpler and more uniform cytology than the vaginal smear and is much less contaminated with bacteria, leucocytes, and histiocytes.

Thus far, approximately 350 specimens of catheterized urine of women have been examined. Of these, 38 were from pregnant women. Cytologic changes characteristic of pregnancy were found in all specimens of this latter group. The earliest specimen examined was of a gestation of 14 weeks and the latest of seven months. A study of earlier as well as of more advanced cases is now in progress. Also, of abnormal conditions resulting from pregnancy, such as abortions, ectopics, hydatidiform moles, chorio-epitheliomas, etc.

The technic used in these studies is similar to the one used in cancer diagnosis. Catheterized urine, varying in quantity from 25 to 100 cc is mixed immediately with equal parts of 95% ethyl or with isopropyl alcohol and centrifuged without much delay for 30 minutes at medium speed. The sediment is spread evenly on a number of slides which have been thinly coated with Mayer's albumen. The smears thus prepared are allowed to stand only for a short time until they begin to show dryness around the edge and then they are immersed in a solution of equal parts of 95% alcohol and ether.

After a fixation of at least 15 minutes the slides are carried through 80%, 70%, and 50% alcohols to distilled water.

The staining is as follows:

Harris Hematoxylin (which has been prepared without acetic acid, and diluted with an equal amount of distilled water) for 6 minutes.

Rinse in distilled water.

Dip in 0.5% HCl (aqueous solution) 6 times.

Place in running tap water for 6 minutes.

Rinse in distilled water, 50%, 70%, 80%, and 95% alcohols.

Stain in OG6 for 1½ minutes.

Rinse in 95% alcohol, 2 changes.

Stain in EA65[†] (procedure No. 267) for 1½ minutes or in EA36[7] (procedure No. 268).

Rinse in 95% alcohol (3 changes); absolute alcohol and xylol, equal amounts; xylol; mount with a coverslip.

*Be sure that smears are never allowed to dry at any time.*

*Summary.* Pregnancy causes distinct morphologic changes in the epithelium of the urinary tract which are reflected in the cytology of the "urine sediment smear." There is an appearance of certain characteristic cell forms such as cells of the "navicular" type corresponding to those described in the vaginal smear of pregnancy. The urine sediment smear provides, thus, a simple, rapid and dependable method for diagnosing pregnancy. It appears to be superior to the vaginal smear in that its cytology shows greater uniformity and is more distinctive. Its practical value as a routine diagnostic procedure and its applicability in the early diagnosis of pregnancy are still to be determined.

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[†] EA65 is the same as EA36 with the difference that the Light Green is one-half strength (0.25% instead of 0.5%). It has the advantage of giving a lighter and more transparent staining which is very desirable in cancer diagnosis. The differentiation between acidophilic and basophilic cells is better with the EA36 (or EA50). This differentiation is more important in vaginal, endocervical, and endometrial smears. Therefore, EA36 (or EA50) is preferable for vaginal, endocervical, and endometrial smears, whereas EA65 is better adapted to other types of smears. Either one of these two stains can be used for all types of smears.

[7] Papanicolaou, G. N., *Science*, 1942, **95**, 438.

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### 16268

## Streptomycin as an Essential Nutrilite.

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In consideration of the mode of action of streptomycin one of the possibilities explored has been that of its functioning as a metabolite antagonist. Little evidence of this has been forthcoming, however, and it was therefore with interest that one read the report of Miller and Bohnhoff[1] on their so-called B variant meningococcus. This variant (mutant) resembled the A variant (mutant) in that both were resistant to, or developed in high concentrations of, streptomycin. The B mutant differed from the A in that it required streptomycin for multiplication either *in vitro*, or *in vivo* in the mouse, while the A mutant did not. The B mutant never emerged in the presence of streptomycin concentrations less than 40 γ/ml, but once secured, its growth was promoted by 5γ/ml but not less. Rare back-mutation to non-streptomycin-requiring forms occurred. A recent paper by Kushnick, Randles, Gray and Birkeland deals with variants (mutants) of *E. coli, P. aeruginosa* and *B. subtilis* requiring streptomycin.[2] All mutants showed this growth stimulation when glucose was present in the medium. In the case of *P. aeruginosa* sodium formate could replace the glucose. The case of *B. subtilis* was unusual in that the streptomycin-requiring mutants grew only under anaerobic conditions. Many papers in the literature[3,4] demonstrate that the antibacterial action of streptomycin is greatly reduced by anaerobic conditions.

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[1] Miller, C. P., and Bohnhoff, M., *Science*, 1947, **105**, 620.

It has been possible to confirm the findings of Miller and Bohnhoff as regards streptomycin-requiring mutants of meningococci. These authors obtained such variants from 16 of 18 strains tested. They do not mention type distribution. In our case the mutants developed with a Type II (No. 763) and a Type II-α strain (No. 1054) but not, in limited tests, with a Type I strain (No. 1168).* The medium we have used is a beef heart, glucose agar containing 4% fresh citrated rabbit blood[5] on which the meningococcus grows readily. On such medium 2 colony forms have appeared similar to those described by Miller and Bohnhoff, but such difference in colony morphology has not been associated in our hands with as sharp differentiation as they found into colonies resistant to streptomycin but not requiring it as contrasted to colonies both resistant to and requiring the antibiotic. Furthermore, our mutant strains showed much less stability than that indicated by Miller and Bohnhoff

TABLE I. Serial Transfers of Mutant Strains of *E. coli.*

| Strains D56 | | | | Strain D57 | | | |
|---|---|---|---|---|---|---|---|
| Transfer No. | Units/ml* streptomycin | Back-mutants† | Colony type | Transfer No. | Units/ml* streptomycin | Back-mutants† | Colony type |
| 1 | 5 | Frequent | Undifferentiated | 1 | 20 | Frequent | Undifferentiated |
| 2 | 20 | ” | ” | 2 | 80 | ” | ” |
| 3 | 80 | ” | Small | 3 | 640 | ” | ” |
| 4 | 80 | None | ” | 4 | 2560 | None | ” |
| 5 | 320 | Frequent | Undifferentiated | 5 | 5120 | Very few | Small |
| 6 | 80 | Very few | Small | 6 | 1280 | Frequent | Undifferentiated |
| 7 | 40 | Frequent | ” | 7 | 320 | ” | ” |
| 8 | 40 | Very few | ” | 8 | 160 | ” | ” |
| 9 | 80 | ” ” | ” | 9 | 40 | ” | ” |
| 10 | 20 | Frequent | Undifferentiated | 10 | 40 | None | Small |
| 11 | 160 | None | Small | 11 | 20 | ” | ” |
| 12 | 160 | ” | Undifferentiated | 12 | 20 | ” | ” |
| 13 | 40 | ” | ” | 13 | 10 | ” | Large |
| 14 | 10 | ” | ” | 14 | 40 | Very few | Undifferentiated |
| 15 | 10 | ” | ” | 15 | 10 | ” ” | ” |

* Concentration of streptomycin in units per ml on which colony grew and from which transfer was made.

† Frequency of colonies on control streptomycin-free medium; *i.e,* reversion to parent type no longer requiring streptomycin as essential metabolite.

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[2] Kushnick, T., Randles, C. I., Gray, C. T., and Birkeland, J. M., *Science*, 1947, **106**, 587.

[3] Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

[4] Bondi, A., Deitz, C. C., and Spaulding, E. H., *Science*, 1946, **103**, 399.

* My thanks are due to Dr. Sara E. Branham of the National Institute of Health, Bethesda, Md., for these strains.

[5] Rake, G., *J. Exp. Med.*, 1934, **59**, 553.

TABLE II.
Optimal Concentrations of Streptomycin for *E. coli* Mutants.

| Strain 56, 6th passage | | Strain 57, 13th passage | |
|---|---|---|---|
| Streptomycin pool III Concentration/ml | Growth on plate | Streptomycin Concentration/ml | Growth on plate |
| 20 u | + | 5 u | 0 |
| 40 u | +++ | 10 u | 3 colonies |
| 80 u | +++ | 20 u | + |
| 160 u | ++ | 40 u | 4 colonies |
| 320 u | 0 | 80 u | 0 |

and apparent back-mutation was too frequent to allow these strains to be used readily as a tool for further study. It may be that such unsatisfactory behavior is due, in part at least, to the fact that colonies for transfer were, after the first few transfers, selected from plates containing high concentrations of streptomycin (800 units/ml or greater).

At the same time that the work with meningococci was started we began investigation of 4 strains of *E. coli* of our own isolation. In limited tests one strain (H36) did not produce streptomycin-requiring mutants on beef heart agar[5] (without rabbit blood), or on a fluid medium of the same beef heart infusion without the agar. The other 3 did. In the case of one strain, D11, there was still a marked tendency to back-mutation although it was less than in the case of the meningococcus mutants. This strain was maintained mostly by transfer from colonies growing at the higher concentrations of streptomycin (3,200 units/ml or greater). Both large and small colony types occurred but again there was no correlation of any such morphological appearance with a demand or no for streptomycin as an essential metabolite.

In the case of the two other strains of *E. coli*, D56 and D57, the later transfers in particular were made from colonies growing on agar with lower concentrations of streptomycin. As can be seen in Table I back-mutation became less frequent and the correlation of small colony with necessity for streptomycin was fairly good. The strains tended towards stability.

It is thus apparent that selection of the streptomycin-requiring mutants in pure form occurs more readily by colony picking from plates of lower, rather than of higher, concentrations of streptomycin. The exact reason for this is not clear but probably these mutants, though relatively resistant to streptomycin, are not as resistant as the mutants which, while growing in concentrations of streptomycin as high as 100,000 units/ml[6] and possibly metabolizing the antibiotic, do do not find it essential for their multiplication. Single cell isolation would be necessary for certainty on this point; its probability is borne out by the fact that the streptomycin-requiring mutants, when plated out on increasing concentrations of commercial streptomycin, or streptomycin A, showed an optimum which for the *E. coli* strains was around 20 to 80 units/ml (Table II). However, resistant forms could readily be selected which grew in much higher concentrations of streptomycin (5,000 units/ml or greater). These, which grow freely in absence of streptomycin, show limitation of growth at higher, but not at lower, concentrations of the antibiotic.

All the above work has been carried out on complex media. It was decided to attempt to adapt the mutants to a synthetic medium. After trial of several, that described by Cohen[7] was finally adopted. For our purposes the pH was adjusted to 7.3 with NaOH before sterilization and a final pH of 7.0 was thus achieved. The medium was used mostly as a broth but also as an agar. Streptomycin-requiring mutants were obtained from both D56 and D57 strains of *E. coli* on such media. For most of the subsequent work D57 mutant ($D57_M$), carried in the fluid medium, has been used. As long as the broth cultures are not held under conditions allowing multipli-

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[6] Lapedes, D., Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 269.

[7] Cohen, S. S., *J. Biol. Chem.*, 1947, **168**, 511.

TABLE III.
Substances* Active as Essential Metabolites for *E. coli* Mutant D $57_M$.

| Active | Inactive |
|---|---|
| Streptomycin A | D-glucosamine HCl |
| Streptomycin B | Streptodine sulfate[11] |
| Dihydrostreptomycin A | Pneumococcus polysaccharides[‖] |
| N-Acetyl streptomycin A [†,9] | *K. pneumoniae* polysaccharides[¶] |
| Streptobiosamine HCl [‡,10] | Streptamine[11] dihydrochloride |
| Lipositol[§] | L-(+)-Arabinose |
|  | N methyl-L-glucosaminic acid[12] |

[*] My thanks are due to Dr. Oskar Wintersteiner and Dr. Josef Fried of the Division of Organic Chemistry, The Squibb Institute for Medical Research, for all the streptomycin and derivative samples; to Dr. H. E. Carter, Department of Chemistry, University of Illinois, and Dr. D. W. Woolley, The Rockefeller Institute for Medical Research, for samples of lipositol; to Dr. J. W. Palmer, Biological Laboratories, E. R. Squibb and Sons, for the polysaccharides of the pneumococcus types and *K. pneumoniae* type B; and to Dr. M. Heidelberger, Department of Medicine, Columbia University, for the polysaccharides of *K. pneumoniae* types A and C.

[†] Contains 15% streptomycin A.

[‡] Activity probably due to residual streptomycin A (see text).

[§] Some preparations showed slight activity (see text).

[‖] Types 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 14, 18, 19.

[¶] Types A, B, and C.

cation to occur for longer than 72 hours back-mutation has been rare and growth has rarely appeared in the control (streptomycin-free) medium within this time. When cultures are held for longer periods, mutants not requiring the antibiotic gradually emerge. For all of the routine passage and selection of mutants, in this work with the synthetic medium, a streptomycin pool designated III has been used. This has the following characteristics: streptomycin A 65%, streptomycin B 30%, other maltol producing material 5%, total streptomycin 64%, inert material 33%, minimal inhibiting concentration (M.I.C.) for *K. pneumoniae* (in the standard biological assay)[8] 0.103 $\gamma$/ml, M. I. C. inhibiting *E. coli* (D57 parent strain) 0.155 $\gamma$, *K. pneumoniae*/*E. coli* ratio 0.67, streptomycin potency 354 units/mg.

Using this strain $D57_M$ the capacity of many different substances to replace the streptomycin pool III in the medium as essential metabolite has been investigated. The results are shown, in summary, in Table III.

One notes that streptidine and streptamine are inactive. Also inactive are the polysaccharide fractions of pneumococci and *K. pneumoniae*. The latter inactivity is particularly to be noted. We also received and tested 3 samples of polysaccharides of *M. tuberculosis* strain H37 kindly supplied by Dr. M. Heidelberger of the Department of Medicine, Columbia University. These represent the high rotating (fraction C), low rotating (fraction $B_{2a}$) and serologically inert (fraction $B_2C$) fractions described elsewhere.[13] All were negative.

As far as the active substances are concerned it is to be noted that lipositol showed very little activity and that such activity as was obtained was not found with every sample. Thus a sample from soybean provided by Dr. D. W. Woolley (see footnote to Table III) was slightly active at 20 $\gamma$ and 40 $\gamma$ per ml but not in lower or higher concentration. A soybean lipositol and a brain lipositol labeled

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[8] Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

[9] Fried, J., and Stavely, H. E., to be published.

[10] Kuehl, F. A., Jr., Flynn, E. H., Brink, N. G., and Folkers, K., *J. Am. Chem. Soc.*, 1946, **68**, 2096; Fried, J., Walz, D. E., and Wintersteiner, O., *J. Am. Chem. Soc.*, 1946, **68**, 2746.

[11] Carter, H. E., *et al.*, *Science*, 1946, **103**, 53; Fried, J., Boyack, J. A., and Wintersteiner, O., *J. Biol. Chem.*, 1946, **162**, 391; Peck, R. L., *et al.*, *J. Am. Chem. Soc.*, 1946, **68**, 29, 776.

[12] Kuehl, F. A., Jr., Flynn, E. H., Holly, F. W., Mozingo, R., and Folkers, K., *J. Am. Chem. Soc.*, 1946, **68**, 536; Wolfrom, M. L., Thompson, A., and Hooper, R. I., *J. Am. Chem. Soc.*, 1946, **68**, 2343.

[13] Heidelberger, M., and Menzel, A. E. O., *J. Biol. Chem.*, 1937, **118**, 79.

TABLE IV.
Relation of Stimulatory and Antibacterial Activity of Different Active Substances.

| | Stimulatory activity on $D57_M$ *E. coli* | Antibacterial activity on D57 *E. coli* |
|---|---|---|
| Streptomycin pool III | 1.0 | 1.0 |
| Streptomycin A | 1.4 | 1.9 |
| Dihydrostreptomycin A | 1.4 | 2.1 |
| Streptobiosamine HCl | 0.06 | 0.07 |
| N-Acetyl streptomycin A | 0.18 | |
| Streptomycin B | 0.7 | 0.54 |

"water extract," containing 3.9% inositol, from Dr. H. E. Carter (see footnote to Table III) were inactive, but a brain lipositol labeled "ether extract," containing 6.3% inositol, from Dr. H. E. Carter was slightly active at 20 γ to 160 γ but not in lower concentration.

The relative growth promoting capacities on mutant $D57_M$ of the substances labeled active in Table III are shown in Table IV. These activities have been referred to that of the streptomycin pool III as unity and are to be regarded as only approximate since the dilutions tested varied from each other by 2-fold. Also shown are the antibacterial activities of these same substances on the parent strain D57 again referred to the activity of pool III as unity. These values are accurate to within ±5%.

It will be noted that the relative values of the growth stimulatory activity agree remarkably well with the antibacterial values of the same substances on the parent strain. The activity of the N-acetyl streptomycin A corresponds to the 15% of streptomycin A which this sample was known to contain. In the case of the sample of streptobiosamine HCl it is not certain whether the activities found are due to a residuum (3.5%) of streptomycin A or whether the compound itself has activity. This is being investigated further. It is of interest that the relative activities of streptomycin B show such good agreement. This would suggest that the same factors may act to decrease the stimulatory activity of streptomycin B relative to A as act to decrease its antibacterial activity on the parent culture. One can perhaps visualize the extra mannose moiety as interfering with the activities of the rest of the molecule.

*Summary*. Streptomycin-requiring mutants were obtained from 2 of 3 strains of meningococci and 3 of 4 strains of *E. coli*. In the case of 2 *E. coli* strains such mutants grew readily on a synthetic medium in the presence of streptomycin. Such mutants appear more stable when isolated from low rather than from high streptomycin concentrations. In the case of the *E. coli* mutants, they exhibit optimal growth at concentrations from 20 to 80 units/ml of a commercial streptomycin or of pure streptomycin A. Growth of the mutants can be promoted by pure preparations of streptomycin A, dihydrostreptomycin A, and streptomycin B and the relative stimulatory activities of these compounds for the mutant agrees closely with the relative antibacterial activities against the parent strain.

My thanks are due to Mr. John Oskay for technical assistance in carrying out these experiments.

### 16269

## Vesicular Stomatitis and Foot-and-Mouth Disease Differentiation by Complement-Fixation.

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It is generally admitted that the differential diagnosis of vesicular stomatitis and foot-and-mouth disease on clinical grounds alone is virtually impossible. Methods of differentiating between the two diseases which have been suggested include: (a) determination of the particle size of the infective agent (Galloway and Elford[1]); (b) determination of infectivity for the developing egg by the chorioallantoic route (Burnet and Galloway,[2] Galloway and Elford[3]); (c) study of the pH stability range of the infective agent (Pyl and Klenk,[4] Pyl,[5] Reppin, and Pyl[6]); (d) tests of susceptibility of several species of animal (*e.g.*, horses, cattle, swine, sheep, guinea pigs) by a variety of routes; (e) cross-immunity tests in experimental animals (Wagener).[7]

In addition may be included epidemiological criteria, such as spreading power and degree of generalization of the disease in infected animals. These are of little use in countries such as the United States of America and Great Britain, where the first object of policy is to limit the possible spread of the infective vesicular diseases of animals by the slaughter of infected stock, and destruction of all material likely to harbour the viruses.

All the methods listed above depend, in the last analysis, on the susceptibility of the test animals, and the virulence of the material used. It is therefore doubtful if too great reliance can be placed on any one method. For example, if, in a test of species susceptibility, one or two horses do not react to inoculation with an unknown virus, which has however infected a similar number of cattle and swine, a diagnosis of foot-and-mouth disease cannot be given with confidence. Similarly, the criterion tentatively adduced by Shahan, Frank, and Mott,[8] depending on the intramuscular inoculation of cattle, has already been found by the same workers to be unreliable, in that an animal inoculated with the virus of vesicular stomatitis has reacted, while observations at this Institute with some strains of the virus of foot-and-mouth disease show that the result of intramuscular inoculation of foot-and-mouth disease virus is not always regular.

In these circumstances, any data which can be gathered rapidly, on an infective agent causing vesicular lesions in cattle and swine, are of great value. The virtues of an *in vitro* test, in respect of speed as much as of economy, are especially attractive. These are the considerations which have stimulated publication of the present paper. Of necessity, since the work has been done in a country where the virus of vesicular stomatitis has not so far been identified in the field, the observations are based on strains which have been passaged in the laboratory; but the well-defined results obtained—better than those usually encountered under comparable conditions with the virus of foot-and-mouth disease—suggest that the method could be of great value with material from field outbreaks. The results presented below support the view that complement-fixation should be added to the list of methods of differential diagnosis, and included in any study of an outbreak of a vesicular disease of cattle or swine.

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[1] Galloway, I. A., and Elford, W. J., *Brit. J. Exp. Path.*, 1933, **14**, 400.

[2] Burnet, F. M., and Galloway, I. A., *Brit. J. Exp. Path.*, 1934, **15**, 105.

[3] Galloway, I. A., and Elford, W. J., *Brit. J. Exp. Path.*, 1935, **16**, 588.

[4] Pyl, G., and Klenk, L., *Zbl. Bakt. Abt.*, 1933, 1, Orig. **128**, 161.

[5] Pyl, G., *Hoppe-Seyler's Z.*, 1934, **226**, 18.

[6] Reppin, K., and Pyl, G., *Arch. wiss. prakt. Tierhlk.*, 1934, **68**, 183.

[7] Wagener, K., *Arch. wiss. prakt. Tierhlk.*, 1933, **66**, 173, 301, 363.

[8] Shahan, M. S., Frank, A. H., and Mott, L. O., *J. Am. Vet. Med. Assn.*, 1946, **108**, 5.

The methods used are still under investigation in a study of the complement-fixation reaction in foot-and-mouth disease. Refinement of technic, however, should lead only to greater delicacy in the detection of immunological differences, a goal greatly desired in respect of foot-and-mouth disease and perhaps not without significance for vesicular stomatitis.

*Methods.* Some details of the strains of virus used are given since other experiments with the same strains have been published by American workers.

*Vesicular Stomatitis* (V.S.) type Indiana Strain USCB, received from Dr. Schoening of the U. S. Bureau of Animal Industry in 1944. It was derived from bovine material from an outbreak in Colorado. It is believed that this strain is identical with that described by Shahan, Frank, and Mott[9] as Ind C. It has been maintained at this Institute by serial passage in cattle, and the material used in the experiments below was of the 8th passage. Epithelium was collected from the tongue of an infected steer on 10th June, 1947, cut up, and dried over sulphuric acid and, later, $P_2O_5$. It was stored in sealed ampoules at 4°C.

V.S. type New Jersey Strain USKP, received as above, and derived from an outbreak in Kansas City, Mo. Later described by the workers cited above as NJM. Two batches of material were used, one of the 3rd passage stored frozen at –20°C from 25th May, 1947, and the other from the 5th passage, dried and stored as the Indiana type material. Guinea pig material of the two type strains was also included, in each case as the 13th serial passage of these strains in guinea pigs, stored as frozen guinea pig pads at –20°C.

*Foot-and-Mouth Disease* (F. & M.) type Vallee "O" Strain ASJ, an Argentine strain of bovine origin. Passaged in cattle at Pirbright from 1944 till 10 April, 1946. Bovine tongue epithelium was collected and stored in 50/50 glycerine and M/25 phosphate buffer at pH 7.6.

F. & M. type Vallee "A" Strain 119, a bovine strain, originating from an outbreak in Great Britain in 1932, and since given 68 serial passages in cattle. Material collected and stored as for "O" type, from 14th October, 1947.

F. & M. type Waldmann "C" Strain 149. Also a bovine field strain, recovered in Great Britain in 1934, and since given 33 serial passages at this Institute. Epithelium was collected on 10th November, 1947, and stored frozen.

Three strains of foot-and-mouth disease, passaged in guinea pigs, were also included in the work. They were: Strain 1, "O" type, passaged at Pirbright since 1924, Strain GB, "A" type passaged since 1929, and Strain GC, "C" type, passaged since 1933.

For use in the complement-fixation test, an approximately 1/10 suspension (calculated on wet weight) was prepared, in 0.9% NaCl for V.S. and in M/25 phosphate buffer for F. & M. The suspensions were prepared from 4-18 hours before use, and then centrifuged at 2,000 r.p.m. for 10 minutes immediately before test. The specific sera were all obtained from guinea pigs which had passed through the disease. In the case of V.S. the infecting strains were those described above, while for F. & M. the strains used were the stock guinea pig strains used in this Institute. The guinea pigs used for V.S. (Indiana) serum had received a subsequent intramuscular inoculation of virus before use as serum donors and those for F. & M. (O, A, and C) serum, a course of 4 inoculations by the same route. The F. & M. sera were lyophilized, but the V.S. sera were stored as fluid sera at 4°C. The complement was a stock lyophilized guinea pig serum prepared in batches for use in routine complement-fixation tests in F. & M. The hemolytic system was obtained from a reputable supplier of biological products.

Details of the test methods will be published in full when the data on F. & M. are presented. Briefly, the procedure is to carry out an abbreviated titration of complement

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[9] Shahan, M. S., Frank, A. H., and Mott, L. O., 1946, *loc. cit.*

TABLE I.
Type-specific Fixation of Complement in Vesicular Stomatitis and Foot-and-Mouth Disease.

| | | Antiserum | | | | | |
|---|---|---|---|---|---|---|---|
| | | Vesicular stomatitis | | Foot-and-mouth disease | | | |
| Antigen | Type | Indiana | New Jersey | “O” | “A” | “C” | Nil |
| Vesicular stomatitis | Indiana | 1.44 | .23 | .24 | .22 | .24 | .14 |
| | New Jersey | .21 | .74 | .30 | .32 | .30 | .17 |
| Foot-and-mouth disease | “O” | .15 | .17 | .37 | .19 | .23 | .18 |
| | “A” | .18 | .20 | .23 | .39 | .24 | .21 |
| | “C” | .17 | .19 | .21 | .20 | .44 | .16 |
| Nil | — | .20 | .16 | .27 | .26 | .26 | .21 |

N.B. The figure in each case represents the amount of complement, as ml of 1/25 guinea pig serum, necessary for 50% hemolysis.

in the presence of the various reagents, combined, and with suitable controls. The doses of complement are arranged in a logarithmic series, and the end-point used is 50% hemolysis, calculated from the readings obtained with the centrifugalized tests on a photoelectric absorptiometer (“Spekker” by Hilger, London). The amounts of the various reagents used are (1) virus, 0.2 ml of 1/10 suspension; (2) serum, 0.4 ml of 1/8 dilution in 0.9% NaCl; (3) complement, 1 ml of a dilution of guinea pig serum in 0.9% NaCl, calculated to give the appropriate dose of a series, *e.g.*, 0.2, 0.3, 0.45, 0.67, and 1.0 ml of a 1/25 dilution. Where a reagent is omitted, the volume is made up with 0.9% NaCl.

Fixation of complement is allowed to take place in a water bath at 37°C for 30 minutes. The hemolytic system is then added (0.5 ml of 3.3% cells with hemolytic included) and incubation continued for a further 30 minutes. The tubes are then removed from the water bath, and centrifuged at 1,500 r.p.m. for 10 minutes. Readings are made directly on the tubes, held in a special mount. From these readings, the per cent hemolysis for each tube is obtained from a graph, and hence the amount of complement for the 50% hemolysis end-point is calculated.

Alternatively, the results may be presented graphically, plotting the per cent hemolysis against the log. dose of complement. Titration of complement alone and in the presence of virus and specific sera gives a sigmoid curve in such graphs, and as it is more convenient to deal with straight lines, the transformation evolved by Gaddum[10] and Bliss[11] is used. The values for per cent hemolysis are converted to probit values, using the tables of Fisher and Yates,[12] and these values are plotted against the log dose of complement. The probit value 5.0 corresponds to 50% hemolysis and the end-points are easily obtained from these graphs.

*Results.* Type specific complement-fixation has been obtained using virus from cattle or guinea pigs in foot-and-mouth disease and from both these sources and also from infected eggs in vesicular stomatitis. Since the results are substantially the same with different antigens, the results of a representative test in which virus of bovine origin was employed is shown in Table I, and, graphically in Fig. 1. The fixation of complement by the homologous virus-serum mixture is demonstrated by the increased amount of complement necessary for 50% hemolysis. In the figure, this is shown by the shift to the right of the appropriate graph.

The result with V.S. Indiana virus is particularly clear-cut. The degree of fixation exceeds by two- or three-fold that hitherto encountered in F. & M. disease, under optimum conditions. The poorer fixation with V.S. New Jersey virus may be explained by the fact that the homologous serum was ob-

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[10] Gaddum, J. H., No. 183, Spec. Rep. Ser., Med. Res. Coun., H. M. Stationery Office, London, 1933.

[11] Bliss, C. I., *Ann. Appl. Biol.*, 1935, **22**, 134.

[12] Fisher, R. A., and Yates, F., *Statistical Tables*, 2nd edit., Edinburgh, Oliver & Boyd, 1943.

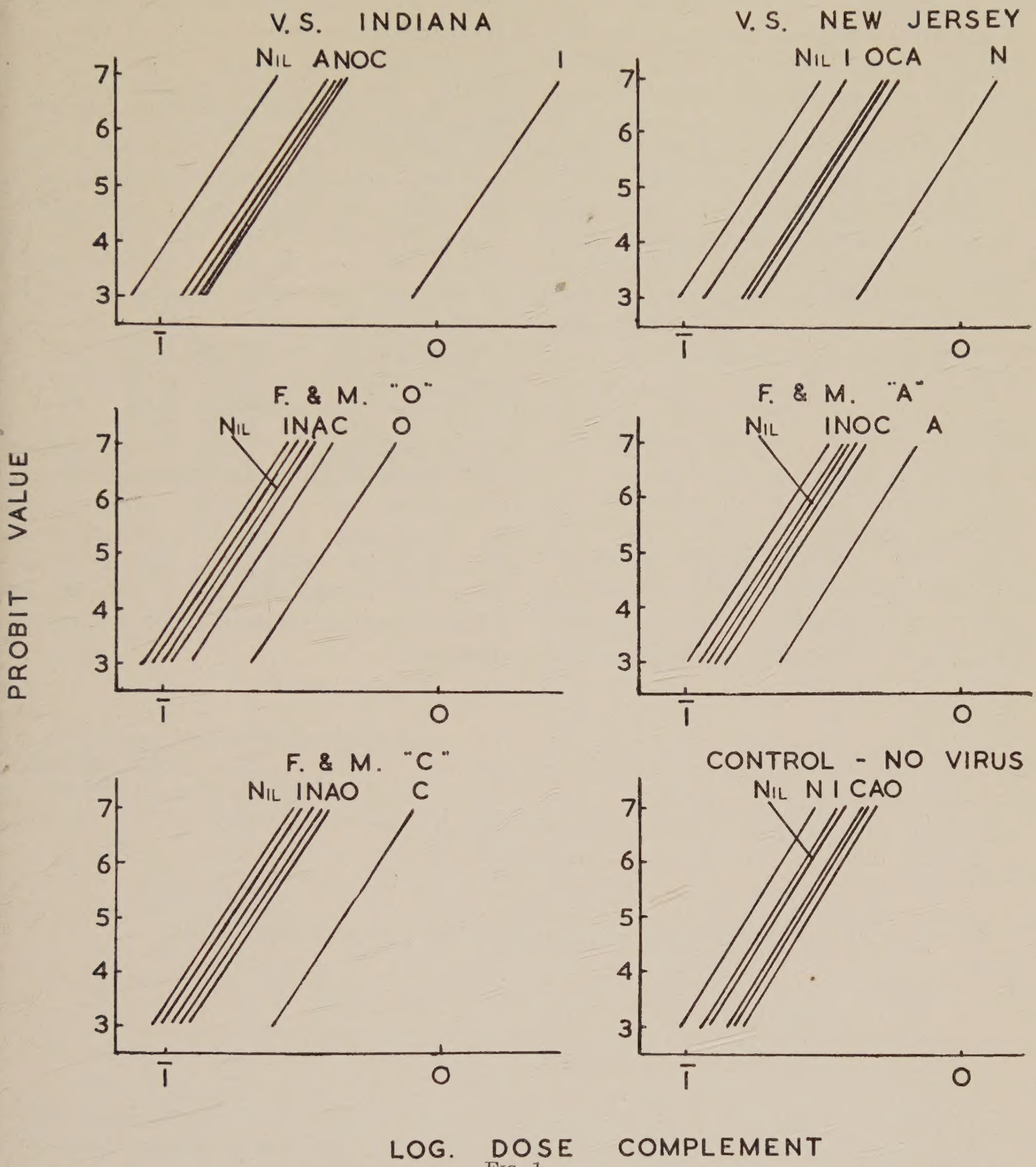

FIG. 1.

Complement fixation in vesicular stomatitis and foot-and-mouth disease. Antisera used are indicated thus: I = V.S. Indiana; N = V.S. New Jersey; O = F. & M. "O"; A = F. & M. "A"; C = F. & M. "C"; Nil = No antiserum (antigen control).

tained from guinea pigs convalescent from the disease, whereas the Indiana anti-serum came from guinea pigs which had had an additional inoculation with virus, by the intramuscular route. That the difference between the results was dependent on the sera was suggested by an experiment where guinea pig and bovine virus of the two types were tested against the same two sera. The results here showed that the degree of fixation recorded with each serum ran parallel in the presence of the different antigens.

The F. & M. type-immune sera were all from guinea pigs which had been hyperimmunized, and yet the difference in the amount of complement necessary for 50% hemolysis, in the presence of homologous as compared with heterologous serum and virus, is only about two-fold. The quantitative difference between V.S. and F. & M. in respect of the complement fixing properties may be correlated with the behavior with neutralizing antibodies. Studies on neutralization of the virus of V.S. by its antisera show that the degree of neutralization recorded for typical sera greatly exceed the corresponding values for F. & M. and its antisera. Thus a 1:10 dilution of V.S. serum may neutralize $10^5$ $I.D._{50}$ of virus (Skinner[13]) while no similar dilution of F. & M. serum has yet been found to neutralize as much as $10^3$ $I.D._{50}$.

*Discussion.* It must be emphasized that the results recorded were obtained with material of only 2 strains of the virus of vesicular stomatitis. Confirmation using other strains is greatly to be desired, in view of the difficulties which have attended the examination of a large proportion of strains of the virus of foot-and-mouth disease from field outbreaks. The bovine V.S. material used was epithelium about 4-6 months old, and 2 of the samples had been dried. It is not known how fresh material, collected, perhaps, at a time when the virus was not fully active, would behave in the test. Nevertheless, in view of the clear-cut results recorded here, an optimistic view of the value of the test is probably justified, and the addition of complement fixation to the armory of tests to which a virus of this group can be submitted would seem to be well worth while.

*Summary.* (1) The fixation of complement by vesicular stomatitis virus and its antiserum is described. (2) This fixation is type specific for the 2 strains of vesicular stomatitis virus of Indiana and New Jersey type available. (3) There is no cross-fixation with any of the three types of the virus of foot-and-mouth disease.

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[13] Skinner, H. H., personal communication, 1947.